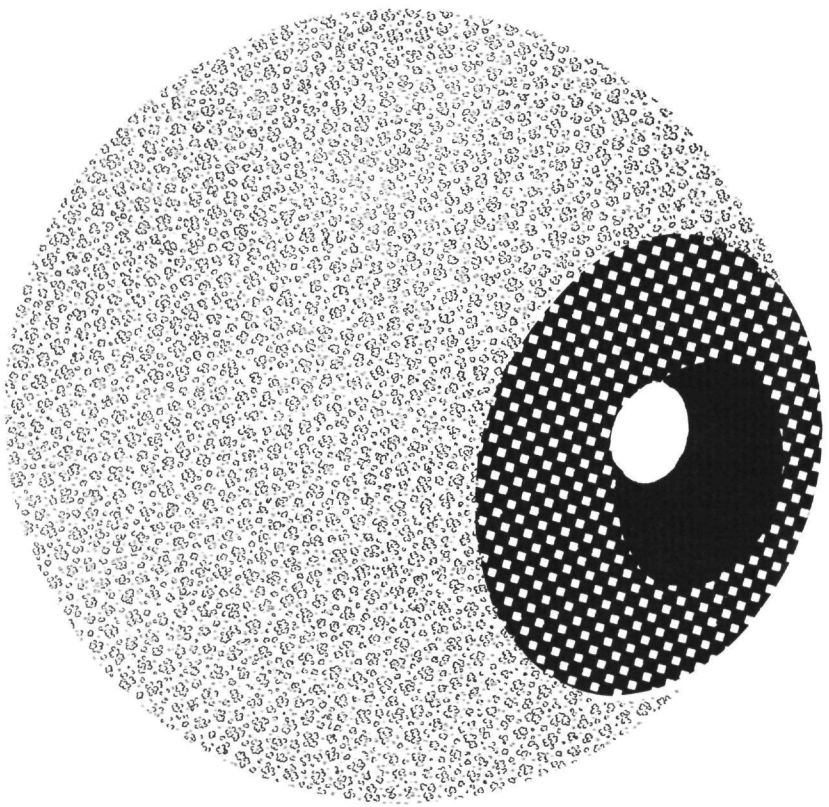
isolation of calf lens messenger rna and its translation in heterologous systems



a.j.m. berns

ISOLATION OF CALF LENS MESSENGER RNA AND ITS TRANSLATION IN HETEROLOGOUS SYSTEMS

PROMOTOR: PROF. DR. H. BLOEMENDAL

ISOLATION OF CALF LENS MESSENGER RNA AND ITS TRANSLATION IN HETEROLOGOUS SYSTEMS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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DER SOCIALE WETENSCHAPPEN,
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IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 22 JUNI 1972
DES NAMIDDAGS TE 2 UUR PRECIES

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THOBEN OFFSET NIJMEGEN

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Aan de nagedachtenis van mijn moeder

Aan mijn vader, Riki, Ruth en .....

CONTENTS

ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine-5′-triphosphate
CMP	cytosine monophosphate
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
E coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetate
GMP	guanosine monophosphate
GSH	glutathione
GTP	guanosine-5′-triphosphate
NCS	Nuclear Chicago solubilizer
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
RNP	ribonucleoprotein
RNase	ribonuclease
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
UMP	uridine monophosphate

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CHAPTER 1

INTRODUCTION

The RNA in a cell originates from transcription of the DNA. The transcriptional products are supposed to undergo a processing in the nucleus and the functional RNA molecules are transported to the cytoplasm. In the cytoplasm there are three different classes of RNA which participate in protein biosynthesis:

1. *Ribosomal RNA (rRNA)*

Ribosomal RNA plays a role in the structure and function of the ribosome. The ribosome of higher organisms contains two high molecular weight RNAs: a 28S RNA (MW circa $1.5 \cdot 10^6$) which is the RNA component of the 60S ribosomal subunit, and a 18S RNA (MW circa $6 \cdot 10^5$) which is the RNA component of the 40S ribosomal subunit. In addition to these two major components smaller RNA molecules are present in the ribosome: a 5S RNA (MW circa $4 \cdot 10^4$) and presumably a 7S RNA. The ribosomal RNA account for the major part of the RNA present in the cytoplasm.

2. *Transfer RNA (tRNA)*

A variety of transfer RNAs is present in the cell. The tRNA is the carrier of the amino acid and the growing polypeptide chain. Each individual tRNA molecule can carry only one kind of amino acid and the different aminoacyl-tRNA molecules are selected by specific trinucleotide sequences in the messenger RNA. A given aminoacyl-tRNA is transiently bound to the ribosome-messenger RNA complex until the amino acid is inserted into the nascent polypeptide. The nascent polypeptide is at all times coupled to the tRNA that has just carried an amino acid in the assembly line.

3. *Messenger RNA (mRNA)*

The term messenger RNA was first used by Jacob and Monod in their interpretation of experiments concerning the synthesis of RNA after phage infection and the kinetics of enzyme induction and repression. Now it is well established that mRNA is the intermediate carrier of information from DNA to protein. The sequence of the nucleotides, of which four types are present in mRNA, determines the order in which the 20 different amino acids are assembled. Each trinucleotide contains the code for one amino acid. This triplet code is recognized by the aminoacyl-tRNA. The stepwise growth of

the polypeptide chain is paralleled by concomitant movement of the ribosome along the messenger.

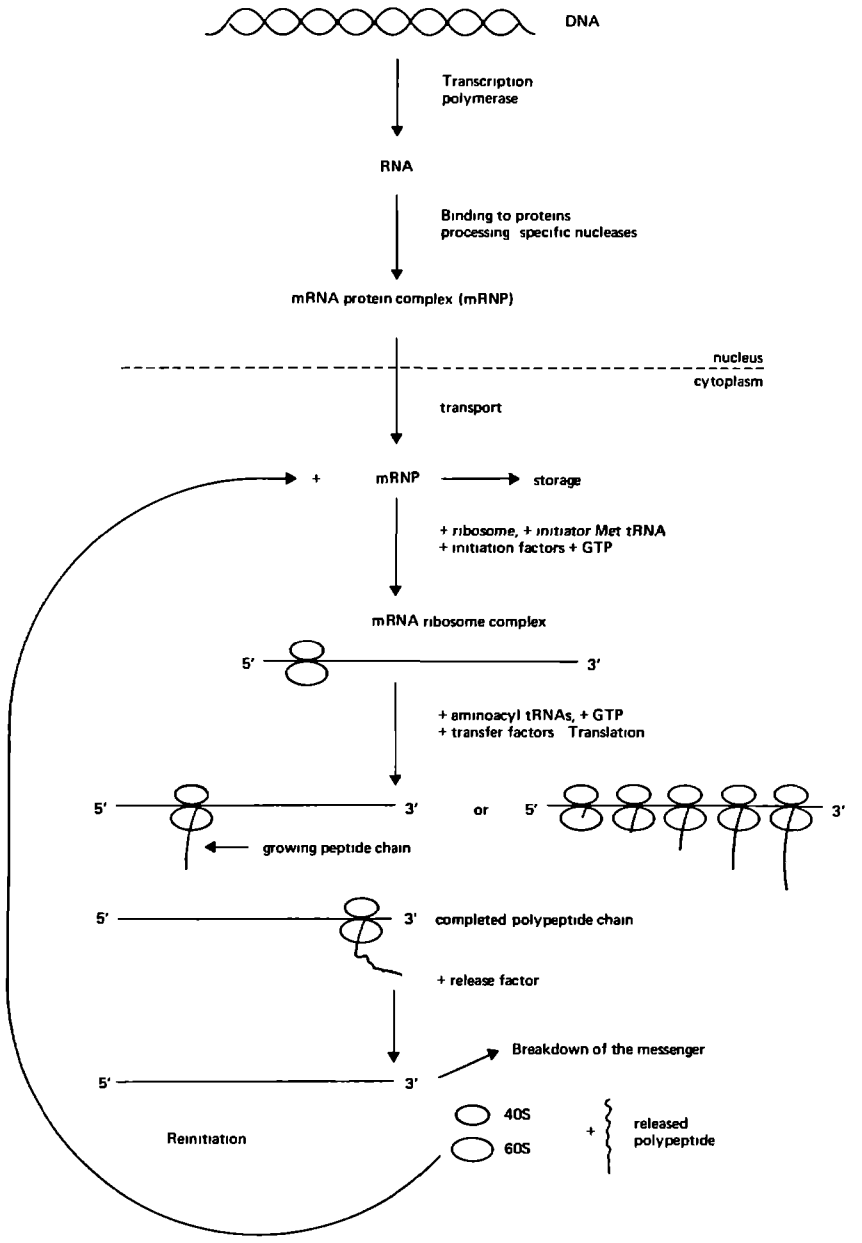
Messenger RNA species exist in a large variety as the synthesis of each protein requires a specific messenger. The bulk of mRNA, which accounts for about 4% of the total RNA present in the cytoplasm has molecular weights from 2×10^5 to 10^6.

Messenger synthesis and messenger function play an important role in the regulation of all processes in the cell. Therefore, the subsequent events from synthesis to degradation of a messenger in an eukaryotic cell will be described (compare also fig.1).

After derepression of a certain genome the DNA is transcribed into RNA by an RNA polymerase. The transcriptional product is believed to be processed partially into functional mRNA molecules[30,98,106,116,133,134,136,138] and transported to the cytoplasm, while it is coupled to protein[71,116,137,147,148]. In the cytoplasm it can be stored[49,144,145,147,156,162], degraded[148] or takes part in protein biosynthesis. In the latter case a complex is formed between the messenger, initiation factors, initiator methionyl-tRNA and the small ribosomal subunit[22,54,59,75,163]. The 60S ribosomal subunit joins the complex and translation starts in the direction 5′ to 3′[135,137]. During this translation the messenger moves through the ribosome, each time offering a following trinucleotide to the tRNA recognition site. For each trinucleotide translated one amino acid is inserted in the polypeptide chain[31]. After completion of the chain a signal on the messenger causes the release of the newly synthesized polypeptide[8,46] and the dissociation of the messenger-ribosome complex, whereafter the ribosome may go into a new cycle[33,41,73,86]. The messenger can be translated again or degraded by RNase.

The availability of purified messengers may contribute to the further elucidation of this whole sequence of events. Its translation in heterologous systems may supply information about tissue and species specific mRNA recognition factors. Hybridization experiments with DNA from different tissues may provide information about gene multiplicity in relation to cell differentiation. The secondary structure and its influence on initiation and translation may be studied[42]. Further it may contribute to the elucidation of posttranscriptional processing and transport mechanisms of messengers.

Until now only the messengers coding for the globin chains[50,89,90,92,100,101,102,111,113,115,122,130], myosin[55], immunoglobin light chain[149], and ovalbumin[132] were isolated and translated in heterologous systems. However, only the globin messengers were purified from contaminating ribosomal RNAs. Also the putative messengers for the histones have


DNA
Transcription
polymerase
RNA
Binding to proteins
processing specific nucleases
mRNA protein complex (mRNP)
nucleus
cytoplasm
transport
+
mRNP
storage
+ ribosome, + initiator Met tRNA
+ initiation factors + GTP
mRNA ribosome complex
5'
3'
+ aminoacyl tRNAs, + GTP
+ transfer factors Translation
5'
3'
or
5'
3'
growing peptide chain
5'
3'
completed polypeptide chain
+ release factor
Breakdown of the messenger
5'
3'
Reinitiation
40S
60S
+
released
polypeptide

been partly characterized[20,43,79], although their translation in heterologous systems has not been reported yet.

The difficulties encountered with the isolation of messengers are threefold:

1. mRNAs are present in only minute quantities as compared with rRNA and tRNA.
2. mRNAs are broken down easily, as they are in general more accessible to nucleases than ribosomal RNA.[166]
3. The bulk of mRNAs exhibits only small differences in molecular weight which renders separation difficult.

In principle, there are several ways to circumvent these problems:

1. The use of a separation procedure with good resolution and high capacity.
2. The selection of a system with low RNase content and the use of RNase inhibitors.
3. a. The isolation of a messenger coding for a very large or small polypeptide. As a relation exists between the molecular weight of the messenger and the molecular weight of the protein, for which it codes, a system synthesizing only a small number of short or long polypeptides will be suitable for the isolation of a single messenger by a simple separation method. Using this procedure a high specialization of the tissue is not a prerequisite. An example might be the albumin messenger from liver.

   b. The messenger can be isolated from a very specialized tissue, which synthesizes only a small number of proteins in relatively high amounts. Such a system yields a relatively high amount of a particular messenger. An example is the reticulocyte, which synthesizes almost exclusively haemoglobin.

   c. Messenger fractionation on the base of information. This can be achieved by antibody precipitation of polyribosomes[4,21,28,36,51,60,64,143,154,165]. The growing peptide chain acts as antigen in this procedure. Although this method seems to be very promising, one encounters a variety of problems as specificity of precipitation is reduced by the interaction of the antibody with the ribosomes. However, this method has been improved by partial digestion of the antibody[65]. Presumably this procedure will be generally applicable in the near future.

In our attempts to isolate a specific messenger a specialized system as described in 3.b. was used. The eye lens which synthesizes mainly α-crystallin[34], a well characterized protein, is such a system. The polyribosomes from

the epithelium and the outer cortex were the source for our messenger preparations. Several RNA fractions were obtained with characteristics of messenger[9]. The definite proof of their messenger function was given by their translation in heterologous systems like the rabbit reticulocyte lysate[11], a cell-free system derived from Krebs II ascites cells[110], and the *in vivo* system of the oocyte of Xenopus laevis.[10]

CHAPTER 2

ISOLATION OF MESSENGER RNA FROM LENS AND RETICULOCYTES

2.1. INTRODUCTION

The difficulty in the isolation of messenger from eye lens polyribosomes is twofold:

a. The messenger comprises only 1–2% of the total polyribosomal RNA.
b. Since the molecular weight of lens polypeptide chains is about 20,000, the sedimentation coefficient of the corresponding messenger can be expected to be about 11S, so that it may be masked by the bulk of ribosomal RNA.

Several separation methods can be used: e.g. sucrose gradient centrifugation or polyacrylamide gel electrophoresis. Although gel electrophoresis enables superb separations, only minor quantities can be fractionated in a single run, which makes this technique less convenient as first separation method. Centrifugation in swinging bucket rotors with sucrose gradients enables the fractionation of larger amounts, but here the separation is less satisfactory and strongly dependent on the amount of material loaded on the gradient. However, the development of zonal rotors made it possible on the one hand to enlarge the capacity significantly, on the other hand to achieve better resolution, as the side wall retardation of sedimenting particles is reduced tremendously.

As will be described below zonal centrifugation with exponential sucrose gradients is a useful technique for the isolation of relatively high amounts of mRNA with a purity which makes further fractionation superfluous.

2.2. ISOLATION OF POLYRIBOSOMES

2.2.1. Isolation of Polyribosomes from Calf Lenses[17]

The eyes of young calves (Dutch pedigree cattle) were used as starting material. After the animals were killed in the slaughter house, the eyes were removed and stored on ice. On the same day the eyes were transported to the laboratory, and the lenses were isolated. All further handling was performed at 0–4°C. After isolation of the lenses the epithelial cell layer was stripped off and 1–2 mm of the outer cortex punched off with the aid of a glass trephine. Homogenization of this material was carried out in one

volume of a medium containing 0.35 M sucrose, 50 mM Tris-HCl, pH 7.4, 25 mM KCl and 5 mM magnesium acetate. A teflon homogenizer was used and ten strokes at about 2000 rpm were applied. The homogenate was centrifuged at 12,000 g for 20 min at 2°C. The supernatant was filtrated through 4 layers of cheese cloth and after the addition of DOC to a final concentration of 0.5% the filtrate was layered on a discontinuous sucrose gradient according to the procedure of Bloemendal et al.[16]. This discontinuous sucrose gradient, prepared in cellulose nitrate tubes of 40 ml, consisted of 9 ml 2 M sucrose and 9 ml of 1.5 M sucrose, both containing 50 mM Tris-HCl, pH 7.4, 70 mM KCl and 5 mM magnesium acetate. Centrifugation was performed at 78,000 g for 12 hours in the rotor 30 of a Spinco preparative ultracentrifuge. After centrifugation the supernatant was sucked off carefully and the pellet, containing the polyribosomes was suspended in a medium containing 50 mM Tris-HCl, pH 7.4, 70 mM KCl and 5 mM magnesium acetate and stored at −70°C until use. By this procedure about 5 mg polyribosomes per 1000 eyes were obtained. It appeared from sucrose gradient centrifugation that the major part of the polyribosome preparations from cortex cells, exhibited an optical density profile with an optimum of 8 ribosomes per polysome, while the epithelial polyribosome preparations contained predominantly monomers with decreasing amounts of di-, tri- and tetrasomes.

2.2.2. Isolation of Polyribosomes from Reticulocytes

Rabbits of 2 à 3 kg were made anaemic by daily injection with one ml 2½% neutralized phenylhydrazine solution during 6 days. Routinely the phenylhydrazine solution was stored at −25°C in the presence of 1 mM GSH. On the 8th day one ml heparin (5000 I.U.) and 0.8 ml 10% nembutal were injected. The animals were bled by hart puncture. The blood, about 70 ml per rabbit, was centrifuged at 2500 g for 10 min. The cells were washed three times with a medium containing 0.13 M NaCl, 5 mM KCl and 7.4 mM magnesium acetate[19]. After every washing and centrifugation the buffy coat was removed as careful as possible. To the cells 4 volumes of 5 mM magnesium acetate was added and after 1 min one volume of 1.5 M sucrose in 0.15 M KCl. This lysate was spun at 12,000 g with the timer set for 10 min and the brake off. If the supernatant was not clear this centrifugation step was repeated. The supernatant was layered over 7 ml 36% (w/w) sucrose in 100 mM Tris-HCl, pH 7.4, 15 mM KCl, and 5 mM magnesium acetate. Centrifugation was performed at 78,000 g for 3 hours in a rotor 30 of the Spinco at 2°C.[67] The pellets were suspended in a medium containing 50 mM Tris-HCl, pH 7.4, 70 mM KCl and 5 mM magnesium acetate and stored at −70°C until use.

2.3.1. Methods

For the isolation of RNA components, polyribosomes, prepared as described in section 2.2., were pelleted at 150,000 g (average) for 2 hours at 2°C in a Ti 50 rotor of the Spinco, suspended in a medium containing 50 mM Tris-HCl, pH 7.4, 6% sucrose and 1% SDS to a final concentration of about 6 mg/ml, incubated at 37°C for 5 min and diluted twice with the same medium lacking SDS.

For the isolation of RNP particles the polyribosomes were pelleted as described above and suspended in a medium containing 50 mM Tris-HCl, pH 7.4, 10 mM EDTA and 6% sucrose to a final concentration of about 3 mg/ml. In order to separate the RNA and RNP particles, obtained in this way, zonal centrifugation with exponential sucrose gradients was applied. The gradients were constructed by pumping a sucrose solution, boiled with 0.02% diethylpyrocarbonate for 30 min and containing 50 mM Tris-HCl, pH 7.4, in a mixing chamber with a fixed volume containing buffer only. Part of the gradient together with the buffer was used as overlayer. Sucrose was added to the sample until the density of the sample was between the highest density of the overlayer and the lowest density of the gradient, from which the first 20 ml was discarded. The sample and the overlayer were forced into the rotor with air pressure up to 0.4 atm. After centrifugation the gradient was displaced from the edge of the rotor at about 20–30 ml/min. The optical density was monitored at 260 nm using a Gilford spectrophotometer equipped with a 2 mm flow cell of IEC. The eluate was fractionated by hand. The different RNA or RNP fractions were precipitated by adding 1/10 volume of 2 M potassium acetate, pH 5.0 and 2.5 volume of cold ethanol. After leaving to stand the solution for 16 hours at −25°C the precipitates were collected by centrifugation and stored under ethanol at −25°C. By this procedure 2 μg/ml RNA could be precipitated quantitatively.

2.3.2. Shape of the Gradients

For the zonal centrifugation exponential gradients were applied, resembling the equivolumetric gradients described by Price[127,128]. The equivolumetric gradient can be computed according to the formula:

$$\frac{r^2}{\eta_m} (\rho_p - \rho_m) = \text{constant},$$

in which r = distance to the center of rotation
ρ_p = density of the particles

ρ_m = density of the medium at r
η_m = viscosity of the medium at r.

With the aid of gradients, obeying this formula, excellent resolution can be achieved. However, the amount of material which can be loaded on such gradients is restricted as with high sample concentrations anomalous zone broadening occurs, depending on centrifugation time. For this reason we designed gradients which were somewhat steeper than the equivolumetric gradient. The details for the construction of several gradients, with examples of separations, are summarized below:

Gradient 1

Application:	Isolation of 9S mRNA from reticulocytes up to 0.5 mg.
Rotor type:	B XXX of IEC.
Gradient:	Mixing volume: 260 ml; sucrose concentration: 31% (w/w). Overlayer + sample volume: 150 ml. 50 ml of the gradient was used in the overlayer. Sample concentration: 3 mg/ml.
Conditions:	Centrifugation at 50,000 rpm for 5–8 hours at 2°C.

This gradient is approximately equivolumetric. The density taken for RNA in 0.05 M Tris-HCl, pH 7.4, was 1.6. An example of a separation with this gradient is shown in fig.1.

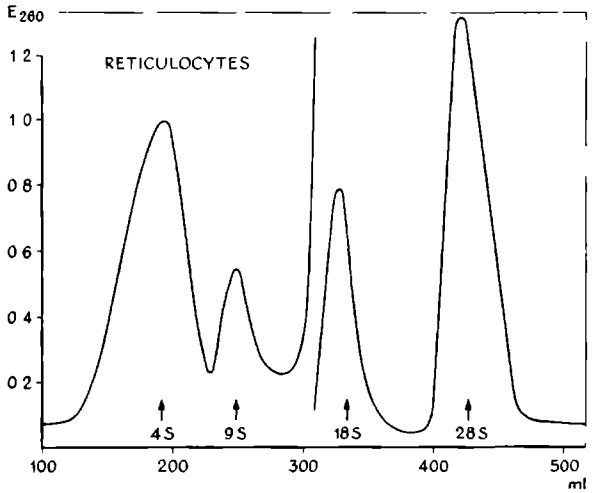


Fig.1. Zonal centrifugation profile of polyribosomal RNA from reticulocytes. 45 mg polyribosomes were applied. Centrifugation was performed for 5.5 hours using gradient 1.

Gradient 2

Application:	Isolation of messengers from lens up to 0.3 mg.
Rotor type:	B XXX of IEC.
Gradient:	Mixing volume: 300 ml; sucrose concentration: 37% (w/w). Overlayer + sample volume: 150 ml. 50 ml of the gradient was used in the overlayer. Sample concentration: 3 mg/ml.
Conditions:	Centrifugation at 50,000 rpm for 12-15 hours at 2°C.

With this gradient a very good separation is obtained in the 8S-16S area. However, even a slight deviation from the described conditions easily results in pelleting of the desired fractions. An example is given in fig.2.

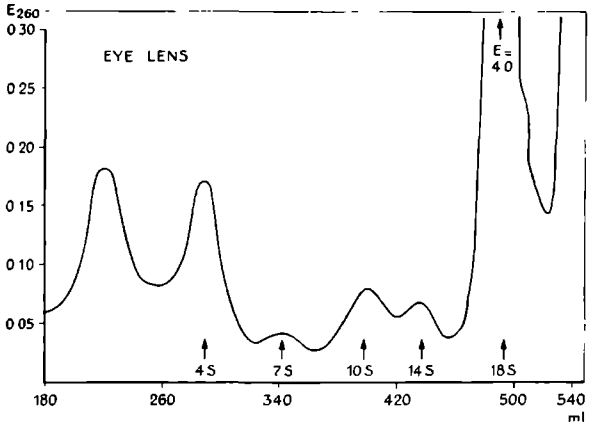


Fig.2. Zonal centrifugation profile of polyribosomal RNA from calf lens. About 25 mg polyribosomes were applied. Centrifugation was performed for 15 hours using gradient 2.

Gradient 3

Application:	Isolation of 9S mRNA from reticulocytes or lens mRNAs up to mg amounts.
Rotor type:	B XXIX of IEC.
Gradient:	Mixing volume: 800 ml; sucrose concentration: 40% (w/w). Overlayer + sample volume: 500 ml. 180 ml of the gradient was used in the overlayer. Sample concentration: 3 mg/ml.
Conditions:	Centrifugation at 35,000 rpm for 20-36 hours at 2°C.

The separation obtained with this gradient is comparable with the separation

obtained with gradient 2. Part of an optical density profile, obtained with this gradient is visualized in fig.18, chapter 4.

Gradient 4

Application:	Isolation of mRNP particles from reticulocyte or lens polyribosomes up to 0.3 mg.
Rotor type:	B XXX of IEC.
Gradient:	All solutions contained: 0.05 Tris-HCl, pH 7.4 and 10 mM EDTA. Mixing volume: 250 ml; sucrose concentration: 28% (w/w). Overlayer + sample volume: 200 ml. 35 ml of the gradient was used in the overlayer. Sample concentration 3 mg/ml.
Conditions:	Centrifugation at 50,000 rpm for 2-4 hours at 5°C.

An example of a separation using this gradient is given in fig.3.

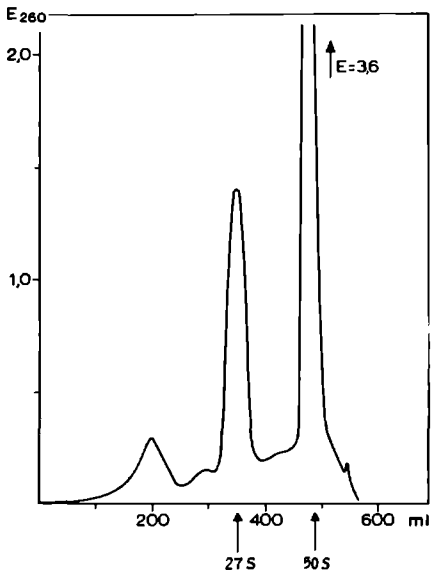


Fig.3. Zonal centrifugation profile of RNP particles from lens polyribosomes. About 20 mg lens polyribosomes were applied. Centrifugation was performed for 135 min using gradient 4.

2.4. THE HOMOGENEITY OF THE SEPARATED RNA FRACTIONS

We tested the homogeneity of the isolated fractions by recentrifugation in

swinging bucket rotors. In fig.4 the recentrifugation profile of the total 9S fraction from the run visualized in fig.1 is shown. There is a slight contamination with 4S or 5S RNA, while no contamination with 18S can be detected. The contamination with 4S and 5S RNA can be reduced easily by applying longer centrifugation times.

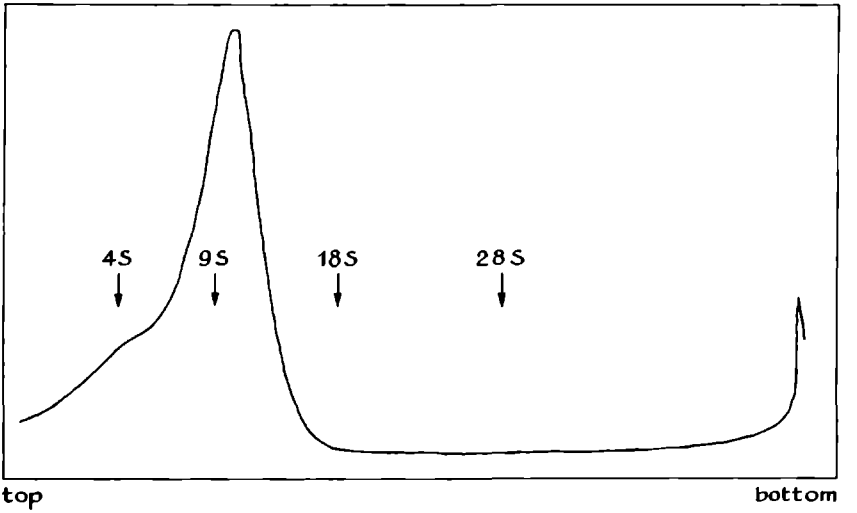


Fig.4. Recentrifugation profile of the 9S fraction from the run visualized in fig.1. Recentrifugation was performed at 41,000 rpm in a SB 283 rotor of IEC at 2°C for 16 hours using a 15-30.2% (w/v) isokinetic sucrose gradient according to Noll. The sucrose gradient was displaced by a heavy sucrose solution pumped through a needle under the gradient. The optical density was monitored with the aid of a LKB optical unit, coupled to a logarithmic recorder.

The recentrifugation pattern of lens RNA fractions are visualized in figures 5, 6 and 7. From these figures it can be seen that the homogeneity of the fractions, corresponding to 7S, 10S and 14S, is better than expected from the optical density profile of the zonal run (fig.2). It appeared that even within a fraction sedimenting as a single band, differences exist. This is demonstrated in section 4.5.2., where the products of subsequent messenger fractions were compared by SDS gel electrophoresis.
The recentrifugation profiles of fractions from gradient 3 are not given, as resolution is comparable with gradient 1 and 2. From gradient 4 recentrifugation profiles are shown in section 3.4., where the different RNP fractions and the RNA derived from them have been compared.

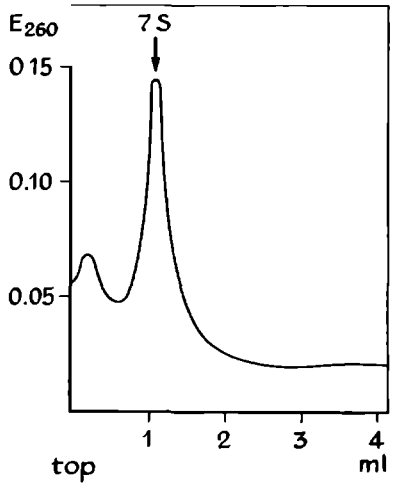


Fig.5. Recentrifugation profile of the 7S fraction from the run visualized in fig.2. Centrifugation was performed at 6°C for 4 hours at 60,000 rpm in a SB 405 rotor of IEC, using an 5-19 1% (w/v) exponential sucrose gradient [117]

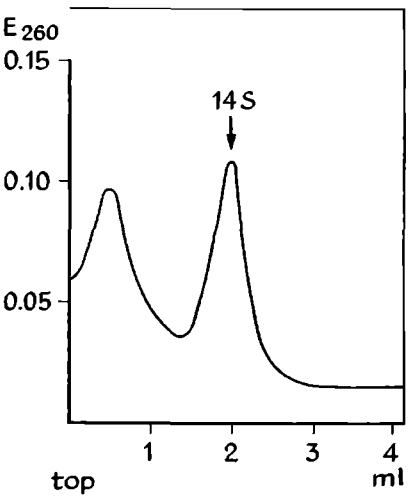


Fig 7. Recentrifugation profile of the 14S fraction from the run visualized in fig.2. Conditions as described in the legend of fig 5.

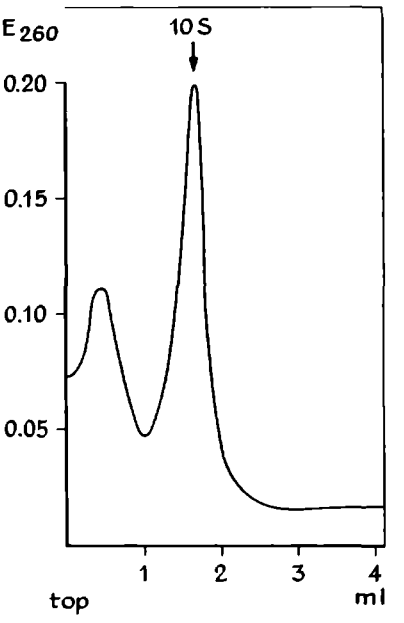


Fig 6. Recentrifugation profile of the 10S fraction from the run visualized in fig 2. Conditions as described in the legend of fig.5.

In this chapter we have demonstrated that the isolation of minor RNA fractions can be carried out efficiently by zonal centrifugation. The great advantages of this procedure are large capacity, high resolution and the possibility of purification in a single step.

CHAPTER 3

CHARACTERIZATION OF THE 10S AND 14S RNA FRACTIONS FROM LENS POLYRIBOSOMES

3.1. INTRODUCTION

There are several criteria by which mRNA can be characterized and distinguished from other RNA species. A number of such criteria are summarized below:

1. Molecular weight
Exact knowledge of the molecular weight of a mRNA is an interesting data, especially in relation to the molecular weight of the protein for which it codes, as it may provide information concerning the length of untranslated regions.[44]

2. Base composition
In general the base composition of mRNAs differs from those of ribosomal and tRNA.[78,105,169] Hence the determination of the base composition can be a simple way to discriminate between mRNA and ribosomal and tRNA. Further it may provide indications about the occurrence of purine or pyrimidine-rich clusters in the untranslated regions of the messenger.

3. Occurrence in RNP particles
mRNA can be isolated from RNP particles with a specific density of about 1.45 in CsCl solutions.[52,94,125] The mRNP particles can be obtained by dissociation of the polyribosomes with EDTA. Generally the mRNP can be separated from the ribosomal subunits by sucrose gradient centrifugation. The mRNA can be extracted and compared with mRNA isolated directly by solubilization with SDS. Comparison of mRNP and mRNA in translation studies may supply information about the function of the adherent proteins.

4. Electron microscopy
The messenger conformation can be studied by electron microscopy.[138] This technique[48,81] may supply information about the single strand character of the messenger and about its length.

5. Stimulation of amino acid incorporation

The stimulatory effect on amino acid incorporation in cell-free systems may be an indication of messenger activity of an RNA fraction[6,58,84,109,146,158,169], while in some cases indications for the synthesis of specific proteins can be obtained by examining the ratio of the different amino acids incorporated. Such incorporation studies were carried out for the characterization of polyribosomes[83,93,103].

Furthermore, it is worth mentioning that in some instances, especially when crude lysate systems are used, an inhibitory effect may also be the result of messenger activity[101] (see also chapter 4).

6. Specific localization

mRNA is localized on polyribosomes. When these polyribosomes are disrupted by low levels of RNase[104,166] or sonication[166], only the messenger is degraded, as it is more accessible to RNase and more susceptable to breakage than ribosomal RNA. Further the messenger should be extractable mainly from polyribosomes and only in minor amounts from single ribosomes[20,39,58,80]. The messenger can also be localized specifically on the small ribosomal subunit after dissociation of the polyribosomes.[63]

7. Hybridization

As all RNA species in normal cells are transcribed from DNA, it should be possible to hybridize them with DNA.[12,78,167,169] With this technique one can demonstrate the relationship between different RNA fractions. This can be achieved by examining the competition effect of other RNA species on the hybridization.[167] It may also give an answer on the multiplicity of genes present in differentiated cells[12,78] (possibly in relation to germ cells), on the stability of stored information during evolution and, when used in combination with DNA fractionation techniques, on the clustering of DNA genomes with identical information.[78]

8. Labelling kinetics

The labelling kinetics of mRNA is different from that of ribosomal RNA. In general mRNA containing fractions, isolated after pulse labelling *in vivo*, can be recognized by their higher specific activity.[24,26,40,58,169] Further the synthesis of mRNA and rRNA can be inhibited specifically by several compounds.[99,123,124,164]

9. Characterization of polypeptides synthesized in a heterologous system programmed with the putative messenger

The characterization of the newly synthesized polypeptides provides evi-

dence for the messenger function of the added RNA fraction. It can also prove the presence of contaminating messengers and show the restrictions of messenger translation in the system used.[50,55,90,92,100,101,102,111,113,115,122,130,132,149]

10. Nucleotide sequence analysis

a. Analysis of certain characteristic sequences, which can be identified easily. It has been shown that a variety of messengers from eukaryotic as well as from viral origin contain poly A tracks,[25,32,38,74,76,95,97] which may have a function in transcriptional regulation,[38] in selection of specific messengers for transport to the cytoplasm[95] and in translational regulation.[153] Therefore the presence of poly A tracks in RNA might be an indication for its messenger character and a means for its classification in a certain group of messengers.
b. The determination of the complete nucleotide sequence. Such a determination is of extreme value as it provides, in addition to the amino acid sequence of the polypeptide, information about initiation and termination sequences and also about the untranslated regions. Until now such analyses have been carried out only with viral RNAs,[1,2,47,114,150,159] which in contrast to non-viral messengers can be prepared highly labelled and in large quantities.

We characterized our 10S and 14S lens messengers by several methods, mentioned above: The molecular weight and the base composition were determined, the presence in RNP particles was demonstrated, the morphology was visualized by electron microscopy and the stimulatory effect on amino acid incorporation was shown. Also the products made under the direction of these messengers were characterized. As this product analysis, carried out after translation of the messengers in different heterologous systems has several special aspects, these latter experiments will be described in separate chapters (chapter 4, 5 and 6).

3.2. MOLECULAR WEIGHT DETERMINATION

3.2.1. Introduction

For the determination of the molecular weight we used the procedure described by Boetker.[18] By using formaldehyde to denature the helical regions of the RNA i.e. to convert the polynucleotide into random coil, it is possible to compute the molecular weight of the RNA from its sedimentation coefficient:

The equation $S_{20,w} = 0.05\ M^{0.4}$ is valid in a medium containing: 1.1 M formaldehyde and 0.09 M Na_2HPO_4 + 0.01 M NaH_2PO_4.

The estimation of the molecular weight by this method seems to be more reliable than using the method of Gierer,[14] where differences in secondary structure may affect the results.

3.2.2. Methods

The RNA (20 μg/ml) was heated at 63°C for 15 min in a medium containing 1.1 M formaldehyde and 0.09 M Na_2HPO_4 + 0.01 M NaH_2PO_4, cooled in ice and either applied onto an isokinetic sucrose gradient, or used for the determination of the sedimentation coefficients with the aid of a Beckman model E analytical ultracentrifuge, adapted with U.V. recording optics. The isokinetic sucrose gradients were identical to the gradients described under figure 4, except that the solutions contained the above mentioned buffer with formaldehyde.

3.2.3. Results

To calibrate our sucrose gradients, the S-value of the 9S messenger from reticulocytes was determined in the analytical ultracentrifuge and applied on reference isokinetic sucrose gradients. The obtained results are summarized in table 1.

TABLE 1

RNA	S-value	MW
4S *E.Coli*	3.0	25,000
9S Retic.	6.6	200,000
10S Lens	7.4	260,000
14S Lens	8.4	360,000

The values are the averages of 2 determinations. The deviation in S-value between 2 determinations was less than 0.2.

The value found for 9S RNA is in good agreement with molecular weight values obtained by others.[44,87,94,168] The molecular weight found for the lens RNA fractions has to be considered as an average molecular weight of a messenger population. As will be shown later, only the 14S messenger seems to be freed from other messenger species, while the 10S messenger fraction contains a number of different messengers with small differences in molecular weight.

3.3.1. Introduction

As we wanted to spend only minor quantities of messenger for base analysis we used a modified method of Katz and Comb. Instead of batch-wise elution of the nucleotides and determination of the absorbance of the whole eluate containing the desired nucleotide, the eluates of the columns were pumped at a constant rate through a flow cell. The absorbance was recorded continuously, and the amount of nucleotide determined by planimetric methods. With this modification it was possible to determine a base composition with 15 μg of RNA.

3.3.2. Methods

The RNA, isolated by zonal centrifugation, was extracted with phenol before hydrolysis. Fractions of the zonal run, precipitated with ethanol, were dissolved in SSA (0.15 M NaCl + 0.015 M sodium acetate) and one volume of water saturated phenol was added. After shaking at 0°C for 15 min the mixture was centrifuged at 5000 g for 10 min, the water layer removed and the phenol and interphase layers reextracted twice with ½ volume of SSA. The combined water layers were reextracted with 2/3 volume of phenol and after centrifugation and removal of the water layer, the phenol was reextracted with ½ volume of SSA. All water layers were combined and the RNA precipitated with 1/10 volume of 2 M sodium acetate and 2½ volume of ethanol at −25°C for 16 hours. The RNA was pelleted and washed once with ethanol, and three times with ether to remove residual traces of phenol. By this procedure 80-90% of the RNA, initially present, was recovered.

The RNA was hydrolyzed in 0.3 M KOH at 37°C for 18 hours in a concentration of about 0.2 mg/ml. The KOH was neutralized with 6 N perchloric acid, the solution was kept at 0°C for one hour, and the precipitate was sedimented at 0°C for 10 min at 5000 g. The supernatant was removed and an equal volume of 0.1 N HCl was added. A solution containing 15-30 μg nucleotides was applied on a Dowex 50X8 (200-400 mesh) column, 10 cm long and 0.5 cm in diameter. The column was eluted successively with 0.05 N HCl and with water. HCl elutes UMP, which is not absorbed, from the column. Water elutes at first GMP, which is followed by AMP and GMP. The latter two nucleotides are separated poorly by this method. Therefore these were applied on a Dowex 1X4 (200-400 mesh) column, 0.5 cm long and 0.5 cm in diameter. CMP and AMP were separated by elution with 0.05 and 0.4 M formic acid respectively.

The eluates of the columns were pumped at a constant rate through a one

cm flow cell of a Gilford spectrophotometer and monitored at 257 and 279 nm. The area under the peaks was measured and used for further calculations. The determination of the absorbance at two wavelengths gave additional information about the purity of the nucleotides.

The extinction coefficients used for the calculations are summarized in table 2.

TABLE 2

Nucleotide	E_{257}	E_{279}	E_{279}/E_{257}
UMP	9600		0.325
GMP	11800		0.565
AMP	14600		0.24
CMP		12500	1.92

3.3.3. Results

The base composition found for the 10S and 14S messenger fractions are summarized in table 3.

TABLE 3

RNA	AMP	UMP	GMP	CMP	$\frac{GMP+CMP}{AMP+UMP}$
18S + 28S	19.4±0.9	18.3±0.5	32.0±0.6	30.3±0.7	1.65
10S	18.9±0.7	30.3±1.0	28.9±2.2	21.9±1.1	1.03
14S	19.2±0.8	30.4±0.6	30.4±0.9	20.0±0.4	1.02
	dAMP	dTMP	dGMP	dCMP	$\frac{dGMP+dCMP}{dAMP+dTMP}$
Calf thymus DNA (27)	27.4	29.5	22.4	20.7	0.78

The values are the averages of 3 to 6 determinations. Standard errors were calculated using the formula: $\sqrt{\Sigma(x-\bar{x})^2/N\times(N-1)}$.

It appears from this table that 18S + 28S RNA have values expected for ribosomal RNA, whereas the 10S and 14S fractions exhibit a more 'DNA-like' composition. The UMP content is higher than normally found for messengers,[92,105,168,169] while the AMP content is lower. The significance of this observation is not clear at the moment. It may suggest that no extended poly A tracks are present, as found in a variety of messenger preparations.[25,32,38,74,76,95,97] Base analyses of messenger fractions which were not subjected to phenol extraction revealed no higher AMP

content indicating that it is unlikely that AMP-rich segments were lost during extraction. On the other hand one should be cautious to conclude that poly U traks are present as UMP is not absorbed by the column and may contain unknown contaminants.

3.4. ISOLATION OF 10S AND 14S RNA FROM RNP PARTICLES

As described in section 2.3.2, mRNP particles containing mRNA can be obtained by treating the polyribosomes with EDTA. As two fractions of messenger-like RNA had been isolated we expected to find at least two different mRNP particles. After zonal centrifugation the mRNP region in front of the small ribosomal subunit was divided into three different fractions. As a reference the mRNP particle obtained after dissociation of reticulocyte polyribosomes was used.

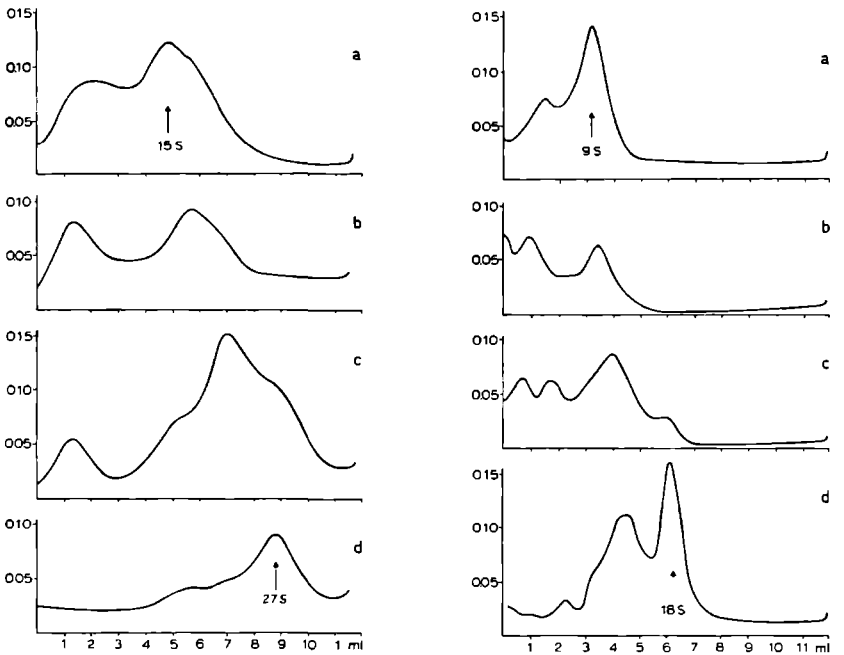


Fig.8. A. Pattern of the RNP particles B. Pattern of the RNA derived from the RNP particles

a) Reticulocyte mRNP, b) Lens first RNP fraction; c) Lens second RNP fraction, d) Lens third RNP fraction. Centrifugation was performed in a SB 283 rotor of IEC under conditions as described in the legend of fig.4.

In fig 8A and 8B the optical density profiles of the RNP and corresponding RNA fractions after sucrose gradient centrifugation are visualized It can be seen that two different RNP particles are obtained from lens polyribosomes The sedimentation values are about 16S and 21S respectively, whilst the corresponding RNA components clearly differed in sedimentation value from ribosomal RNA

Some breakdown of RNP particles seems to occur as both reticulocyte and lens RNP preparations contain some low molecular-weight contaminants. Part of the low molecular weight components may be composed of 5S RNA and protein, a complex which can easily be found in the mRNP area.[94] This is supported by the observation that the contamination with low molecular weight material is less in the heavier fractions

The 16S and 21S RNP particles did not possess the expected density in CsCl gradients, in contrast with the 15S mRNP from reticulocytes In several experiments a density of about 1 53 was found, which is close to the density of the small ribosomal subunit This discrepancy may have its base in the isolation procedure of the polyribosomes from lens. It is known that DOC is able to remove proteins from the polyribosomes, which are identical to protein components present in the mRNP particles.[118] Therefore DOC may have stripped proteins from the mRNP, by means of which the density has been raised.

3 5. ELECTRON MICROSCOPIC INVESTIGATION

The 10S mRNA was investigated with the aid of electron microscopy The RNA was spread on carbon film in the presence of urea, fixed with 1% glutaraldehyde and carbon platinum shadowing was applied with an angle of 15 degrees[48,81] The thickness of the threads measured in the shadowed preparations is consistent with the average thickness reported for single stranded RNA. The length of the threads varies from 0.3 to 1 μm (fig 9). Occasionally longer strands were found, which possibly resulted from the association of the smaller pieces (fig.10) In fact the longer filaments show different width and branching.

It was not possible to determine the unity of length of the messengers from these micrographs.

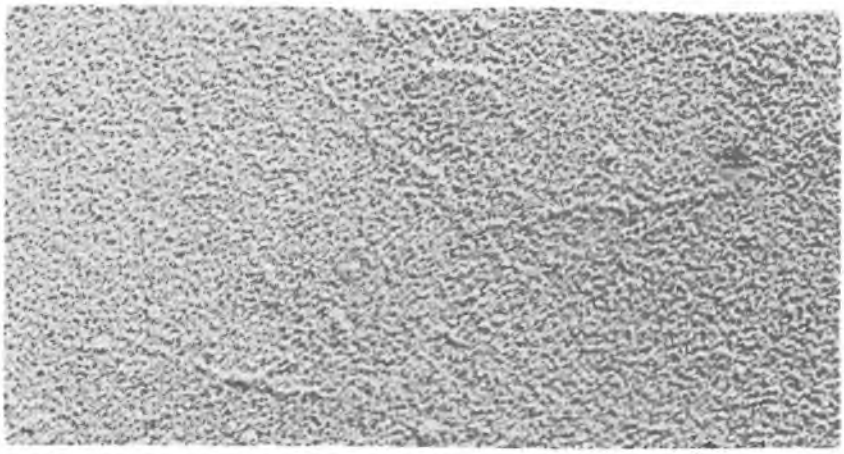


Fig.9. Electron micrograph of lens 'messenger' RNA fractions. Magnification 100,000. The major strand is apparently built up by shorter segments joined together.

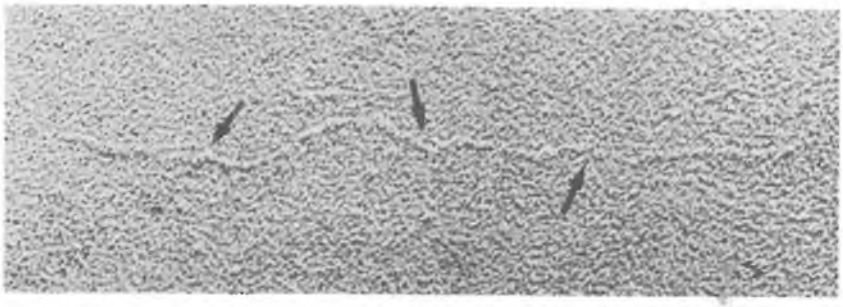


Fig.10. The same preparation showing a longer strand apparently built up by shorter segments. (The arrows indicate overlapping regions.)

3.6. STIMULATION OF AMINO ACID INCORPORATION BY THE 10S AND 14S MESSENGER FRACTIONS

It seems reasonable to assume that protein biosynthesis on ribosomes, depleted from endogenous mRNA may be stimulated by the addition of messengers from other sources, whereas extraneous ribosomal RNA should not influence the amino acid incorporating ability. In order to verify this assumption a number of cell-free incubations was carried out.

Incubations in a total volume of 250 μl were performed at 37°C for 30

min The incubation mixture contained 200 μg ribosomes from reticulocytes, preincubated at 37°C for 90 min according to Cohen, 700 μg 100,000 g supernatant protein, 2 μg RNA, 20 μl 0.6 M KCl wash (containing the wash of 300 μg polyribosomes from reticulocytes), 0.05 M Tris-HCl, pH 7 4, 0.15 M KCl, 3 6 M magnesium acetate, 1 mM ATP, 0.2 mM GTP, 3.2 mM phosphoenolpyruvate, 5 μg pyruvate kinase, 5 mM 2-mercaptoethanol, 0 05 mM L-amino acids except Leu and 0.05 mM DL (^{14}C) Leu (spec act 55.2 mCi/mmole). The reaction was terminated by the addition of one volume 10% TCA. The hot TCA precipitable material was counted in a liquid scintillation counter The effect of the addition of the RNA fractions is visualized in table 4.

TABLE 4
Stimulation of amino acid incorporation by RNA fractions from lens and reticulocyte polyribosomes

Added fraction	Incorporation of Leu (pmoles)	
	- KCl Wash	+KCl Wash
–	2 7	3 3
18S	3 3	3 3
9S reticulocytes	6 5	7 6
10S lens	7 6	12 2
14S lens	7 6	10 7

From this table it can be concluded that the addition of messenger to the cell-free system results in a stimulation of the amino acid incorporation, while the addition of ribosomal RNA has no influence. This excludes the possibility of non-specific stimulation by high molecular weight RNA Crude initiation factors from reticulocytes enhance the stimulation, which might indicate that initiation takes place [112,129] However one should be very careful with the interpretation of this effect, as the KCl wash contains a whole spectrum of factors needed for protein biosynthesis

3.7 DISCUSSION

Some properties of the messengers from lens have been described The molecular weights were found to be 260,000 and 360,000 for the 10S and 14S messenger fraction, respectively, which appears to be an interesting finding

in relation to the molecular weight of the corresponding polypeptide chains. This will be discussed in chapter 4.

The base composition of both messenger fractions differed significantly from the base composition of ribosomal RNA, indicating that it is unlikely that these fractions are contaminated with considerable amounts of fragmentation products of ribosomal RNA. Moreover, since the found AMP content is rather low, the presence of large poly A tracks seems unlikely.

The occurrence of the 10S and 14S messengers in RNP particles, obtained after dissociation of the polyribosomes with EDTA is consistent with observations described for the reticulocyte system.[23,94,155] However the density of the lens mRNP particles was found to be higher than that reported for particles from other sources.[23,52,118,125,148] This may be caused by the use of DOC in the isolation procedure of the polyribosomes. It is known that DOC may remove proteins from the polyribosomes.[118]

Electron microscopy confirmed the expected single strand character of the 10S messenger fraction. However it was disappointing that no well-defined size could be determined from the micrographs, due to the tendency of the RNA to associate. Whether this association is related to special sequences at the 3′ and 5′ ends or to artefacts by the preparation for electron microscopy has still to be elucidated.

The stimulation of the amino acid incorporation by these RNA fractions was likewise in accordance with their messenger function. The 3 to 4 fold stimulation is of the same order of magnitude as generally found for messenger stimulation in preincubated eukaryotic cell-free systems.[111,115] It should be kept in mind that in crude non-preincubated lysate systems often an inhibition of the amino acid incorporation is frequently observed.[100,101] Double stranded RNA structures in the messenger molecule are claimed to be responsible as these structures seem to inhibit the initiation process.[68]

We may conclude that all properties summarized above are in agreement with the messenger function of the 10S and 14S RNA fractions. The definite proof of this function will be given in the subsequent chapters.

CHAPTER 4

TRANSLATION OF LENS MESSENGERS IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM

4 1. INTRODUCTION

The appearance of a new specific polypeptide in a cell-free system after incubation with an exogenous messenger, proves that the added RNA possesses a messenger function Moreover it demonstrates that the heterologous cell-free system is able to translate the foreign messenger

There are a few reports on eukaryotic messenger translation e g. the translation of the myosin messenger on reticulocyte ribosomes[56] and the translation of globin messenger on rat-liver ribosomes[130], the authors of both reports provide evidence that tissue specific factors are involved in messenger translation Such specificity is also reported for T_4 infected *E coli* The initiation factor F_3 can be fractionated into components, exhibiting different activities towards MS2 and T4L RNA [35,82,96,127] This would mean that a messenger from one tissue (or species) cannot be translated in another system without the addition of tissue (or species) specific factors This inability might be due to tissue or species specific recognition sequences on the messenger which cannot interact properly with the specific initiation factors from the foreign tissue or species According to this hypothesis addition of both messenger and initiation factors from the same tissue (or species) would result in the translation of the message

In contrast herewith are several reports which provide evidence against such stringent specificity. Lockard and Lingrel demonstrated that the 9S messenger from mouse reticulocytes is translated in the rabbit reticulocyte cell-free system. However these experiments only prove that the species barrier need not to be an absolute hindrance. Almost the same argument is valid for the observed translation of immunoglobulin messenger in a reticulocyte lysate as there is a narrow relationship between the tissue from which the messenger is extracted and the reticulocyte cell-free system in which it is translated.[149] On the other hand, the translation of reticulocyte messenger in the living oocyte of Xenopus laevis[90] and in a cell-free system derived from Krebs II ascites cells[111] provide more convincing evidence against the general occurrence of stringent tissue- and species specificity These systems, however, are rather undifferentiated and may therefore possess a variety of initiation factors for the translation of their own messengers It is conceiv-

able that one of these factors would be able to recognize the foreign messenger. In view of these considerations tissue specific factors would be expected to prevent the translation of exogenous messenger from a specialized tissue in a system derived from another specialized tissue of another species. However, if translation occurs this would provide evidence against a stringent tissue and species specificity.

In this chapter we shall provide this type of evidence, in case of lens messenger translation, by identifying the products made in a rabbit reticulocyte cell-free system programmed with calf lens messengers. The translation of the lens messengers in the reticulocyte lysate will further yield data about the origin of the N-terminal methionine and the presumably general nature of the acetylation mechanism, which is responsible for the acetylation of the N-termini of the α-crystallin polypeptide chains.

4.2. THE CHEMISTRY OF LENS PROTEINS

For the identification of newly synthesized polypeptide chains it is necessary to know a number of characteristics of the native protein. As it has been shown that in adult bovine lenses both epithelial and cortex fiber cells are highly specialized for the synthesis of α-crystallin[34] we shall restrict ourselves chiefly to the description of this protein.

α-Crystallin is a protein with an average molecular weight of about 800,000 daltons[15] which is composed of aggregated polypeptide chains. Four different polypeptides occur: two acidic (αA_1 and αA_2) and two basic (αB_1 and αB_2) chains.[140,160] The amounts in which they are present in the aggregate decrease in the order αA_2, αB_2, αA_1 and αB_1.[15] The αA_1 chain is almost identical to the αA_2, as has been shown by fingerprinting techniques.[120] It has been postulated earlier that the αA_2 polypeptide is a precursor of the αA_1 chain. If this is true we do not expect to find any αA_1 after the addition of lens messengers to heterologous systems.

The molecular weight of αA_1 and αA_2 appeared to be approximately 19,000, while the molecular weight of αB_1 and αB_2 was estimated at 22,000 by SDS gel electrophoresis and several other techniques.[5]

The α-crystallin polypeptides can also be characterized on the base of charge by gel electrophoresis on acidic and basic urea gels.[142] Electrophoresis on acidic urea gels resolves the polypeptides of α-crystallin into two groups of polypeptides, αA and αB, whereas electrophoresis on basic urea gels resolves all four polypeptides.

A number of characteristic amino acid sequences are also known. The

most interesting is the N-terminal sequence, which is identical for all four polypeptide chains: N-acetyl-Met-Asp-Ile-Ala.[62,141] Both the acetyl group and the methionine are interesting aspects for the study of protein biosynthesis, particularly in view of the elucidation of the initiation mechanism. The N-terminal tetrapeptide can easily be obtained by subtilisin digestion of α-crystallin,[62] while its separation from the bulk of other peptides can be achieved by using a Dowex 50 column which does not absorb peptides without free NH_2 groups. The N-terminal dipeptide: N-acetyl-Met-Asp can be obtained by digesting the polypeptides with pronase.[61] Both the tetrapeptide and the dipeptide can be identified by high voltage electrophoresis.[62]

In the αA_2 chain two methionine residues are present. After aminoethylation of the αA_2 polypeptide, trypsin releases two methionine containing peptides which can be identified by paper chromatography.[120]

The other water-soluble structural lens proteins, the β- and γ-crystallins, are not characterized so well. Their molecular weights are in the region of 25,000 daltons. The β-crystallins are acetylated too, although, presumably, they contain no methionine residue in N-terminal position.

4.3. METHODS

4.3.1. Preparation of the Rabbit Reticulocyte Lysate[3]

Rabbits were handled and reticulocytes were washed as described in section 2.2.2.. To the washed cells one volume of cold distilled water was added and the lysed cells were centrifuged at 30,000 g for 10 min at 2°C. The supernatant was recentrifuged. The lysate was divided into 0.3-1.0 ml fractions, frozen in liquid nitrogen and stored at −70°C until use. The lysate, containing about 150 mg protein/ml could be kept active for several months.

4.3.2. Incubation Conditions[101]

Assays for messenger translation were performed in a volume of 0.5 ml at 37°C for 75 min in a medium containing: 200 μl reticulocyte lysate, 100 mM ammonium acetate, 0.01 M Tris-HCl, pH 7.4, 2 mM magnesium acetate (in addition to the concentrations of salts present in the lysate), 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 30 μg creatine phosphokinase, 100 μM of all amino acids except methionine, 5 μCi (^{35}S) Met (spec.act.: 10-25 Ci/mmole) or 0.5 μCi formyl- (^{35}S) Met-$tRNA_f^{Met}$ and 10 μg messenger RNA. After incubation 25 μl 0.25 M EDTA, 10 μg pancreatic RNase and 150 μg α-crystallin was added and the mixture was kept 30 min at 37°C. The mixture was either precipitated with TCA or used directly for the prepara-

tion of globin.

Precipitation was achieved by adding one volume of 10% TCA. The precipitate was washed three times with 5% TCA and once with ethanol, ethanol: ether (1:1 v/v) and ether respectively. The obtained material was digested with subtilisin or pronase.

4.3.3. Preparation of Globin

Globin was prepared from reticulocyte lysate mixtures by adding the suspension dropwise to 15 volumes 2½% oxalic acid in acetone at 0°C under vigorous mixing. When globin was prepared from incubation mixtures for assaying lens messengers, total lens crystallin (1 mg/ml incubation) was added as carrier before the acidic acetone precipitation was carried out. The acetone precipitate was centrifuged at 3000 g for 5 min, washed three times with cold acetone and once with ether. The precipitate was dried at room temperature.

4.3.4. Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis according to Weber and Osborn was performed in 8.0X0.5 cm cylindrical glass tubes at 5 mA/gel for 12 hours. The gel contained: 12.5% acrylamide, 0.333% N,N'methylene-bisacrylamide, 0.05% TEMED, 0.4 mg/ml ammonium persulphate, 0.1% SDS, 0.1 M sodium phosphate, pH 7.0. The electrode buffer consisted of 0.1 M sodium phosphate, pH 7.0, 0.1% SDS and 1% 2-mercaptoethanol. The gels were preelectrophoresed for one hour at 5 mA/gel.

The sample was solubilized in 1% SDS, 1% 2-mercaptoethanol and 0.01 M sodium phosphate, pH 7.0 and heated at 100°C for 3 min. When the sample contained globin, amounts up to 200 μg per gel were applied. After electrophoresis the gels were stained with Coomassie blue for 2 hours. The Coomassie blue solution was prepared by adding 1.25 g Coomassie blue to 454 ml 50% methanol and 46 ml acetic acid. The gels were destained at 37°C in a medium containing 250 ml methanol and 75 ml acetic acid per liter. If staining was too faint the staining procedure was repeated.

Electrophoresis on acidic urea gels was performed according to Schoenmakers et al.[142] in 8.0X0.5 cm cylindrical glass tubes at 4 mA/gel for 3½ hours. The gels contained: 10% acrylamide, 0.2% N,N'methylene-bisacrylamide, 0.006% potassium metabisulphite, 0.015% ammonium persulphate, 6 M urea and 0.25 M formic acid. Polymerization was performed at 40°C for 2 hours. The electrode buffer contained: 0.25 M formic acid, 6 M urea and 1% 2-mercaptoethanol. Preelectrophoresis was carried out at 5 mA/gel for one hour. Samples were solubilized in electrode buffer, containing 10% sucrose and applied to the gel in amounts up to 400 μg.

After electrophoresis the gels were stained with Amido Black for 30 min. The Amido Black solution was prepared by adding 2.5 g Amido Black to 250 ml methanol, 35 ml acetic acid and 215 ml water. The gels were destained electrophoretically in 2% acetic acid.

Electrophoresis on basic urea gels was performed according to Schoenmakers et al.[142] in 8.0X0.5 cm cylindrical glass tubes at 4 mA/gel for 3.5 hours. The gels contained: 10% acrylamide, 0.2% N,N'methylene-bisacrylamide, 0.006% TEMED, 0.004% potassium ferricyanide, 0.07% ammonium persulphate, 0.075 M Tris-, 0.0018 M EDTA-, 0.0084 M boric acid, pH 8.9, and 6 M urea. The electrode buffer contained: Tris-EDTA-boric acid (as mentioned above), 6 M urea and 1% 2-mercaptoethanol. Gels and buffers were prepared shortly before use. Preelectrophoresis was performed at 5 mA/gel for one hour. Samples were applied in electrode buffer, containing 10% sucrose in amounts up to 400 μg. Staining and destaining was carried out as described for the acidic urea gels.

Preparative gel electrophoresis was performed in 9.0X1.3 cm cylindrical glass tubes at 10 V/cm for 14 hours. The gels were prepared as described above. Samples containing 5 mg protein were applied to these gels. After electrophoresis a small slice of the gel was stained. The area containing the desired polypeptide was cut out, minced, dialysed against distilled water, containing 5 mM 2-mercaptoethanol, and after filtration to remove the gel the filtrate was lyophillized.

The radioactivity in the gels was determined in two ways:

1. After monitoring at 540 nm in a Gilford spectrophotometer, adapted with a gel scanner, the gels were cut into 1.5 mm slices, which were solubilized with 200 μl NCS at 60°C for 16 hours and after addition of a toluene based scintillator counted in a Packard liquid scintillation counter.
2. The gels were sliced longitudinally, dried down under vacuo on filter paper and autoradiographed with a Kodak X-ray film type RHP-Royal X-omat.

4.3.5. Peptide Analysis

Tryptic Digestion

Tryptic digestion was carried out only on the αA_2 polypeptide chains. αA_2 chains were separated from the bulk of the other polypeptides by gel electrophoresis on basic urea gels.

The gel segment containing the αA_2 chains was cut out and handled as described in section 4.3.4.. The polypeptides were aminoethylated by the method of Raftery and Cole: The polypeptides were solubilized in a solution

containing 8 M deionized urea, 0.5 M Tris-HCl, pH 8.6, 2 mg/ml EDTA and 1.3% 2-mercaptoethanol. Before adding the protein this solution was fluxed with argon during 5 min. The dissolved protein (about 15 mg/ml) was left for 2 hours at room temperature and 25 μl/ml ethylenimine was added. After aminoethylation for two hours at room temperature the polypeptides were precipitated with 15% TCA. The TCA was removed by washing with acetone and the material was dissolved in 0.1 M ammonium bicarbonate, pH 8.9 and digested with trypsin (1:50 w/w) for 5 hours at 37°C. The resulting peptides were lyophillized, dissolved in water and subjected to descending paper chromatography on Whatman 3 MM paper eluted with butanol-acetic acid-pyridine-water mixture (60:12:48:40 v/v). Tryptic peptides from (^{35}S) Met-labelled αA_2-crystallin, prepared by *in vitro* incubation of a lysate from calf lens were used as markers. These markers have been proven to have the same chromatographic behaviour as the native methionine containing peptides of αA_2.[120]

After chromatography for 18 hours the chromatogram was dried and cut into one cm strips, which were counted in a liquid scintillation counter using a toluene based scintillator.

Pronase Digestion

Pronase digestion was performed in a solution of 0.1 M ammonium bicarbonate and 1 mM $CaCl_2$ for 6-24 hours at 37°C as described earlier.[61] If no complete solubilization was obtained a second pronase treatment was performed. After digestion the peptides were either lyophillized or treated with Dowex 50 in order to remove the unblocked peptides. For this treatment 3/4 volume of a Dowex suspension was added, mixed thoroughly and centrifuged. The supernatant was removed and the resin washed with one volume of distilled water. After centrifugation the two supernatants were combined and lyophillized.

The residual peptides were dissolved in distilled water and subjected to paper electrophoresis on Whatman 3 MM paper in acetic acid-pyridine-water (6:200:794 v/v), pH 6.5. Radioactivity on the electropherogram was determined as described for tryptic peptide analysis. Reference peptides were stained for methionine using platinic iodide.[37]

Subtilisin digestion

Subtilisin digestion was performed in 0.1 M ammonium bicarbonate at 37°C for 6-16 hours.[62] Further handling was identical as described for pronase digestion.

4.4. IDENTIFICATION OF THE POLYPEPTIDE MADE UNDER THE DIRECTION OF THE 14S MESSENGER FROM LENS

4.4.1. Analysis of the N-Terminal Peptide

As all polypeptides of α-crystallin contain the N-terminal sequence N-acetyl-Met-Asp-Ile-Ala we examined first whether the N-terminal dipeptide could be detected in the reticulocyte lysate after incubation in the presence of 14S lens messenger. As we expected that no N-terminal acetylation would occur in the rabbit reticulocyte cell-free system (^{35}S) Met-tRNA$_f^{Met}$, deriv-

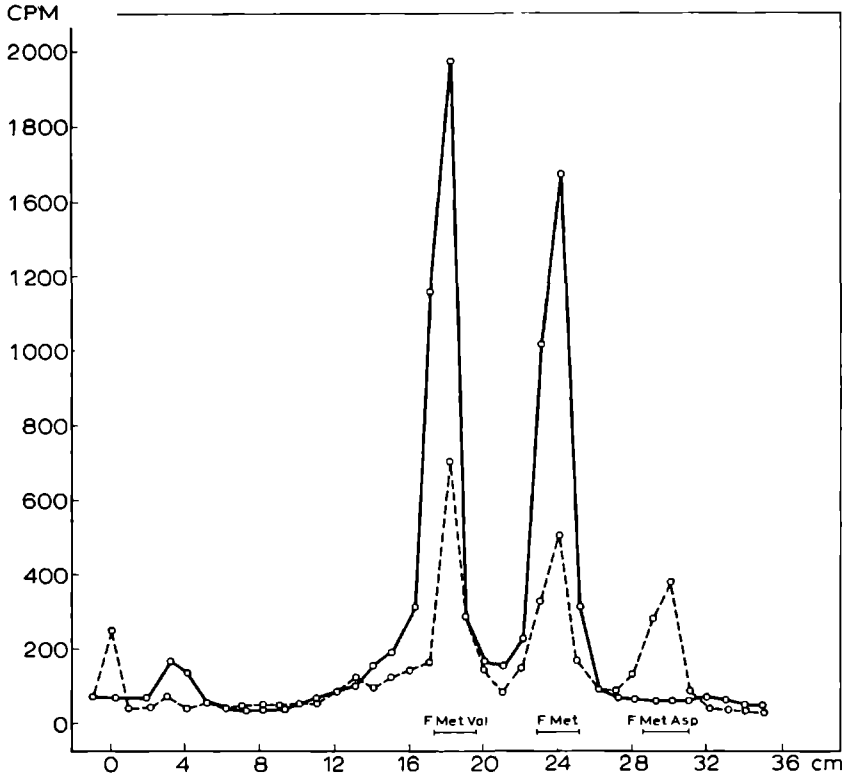


Fig.11. Paper electrophoretic analysis of the N-terminal dipeptide of polypeptides, synthesized under the direction of the 14S lens messenger. The peptides, obtained from a 100 µl incubation mixture were digested with pronase and treated with Dowex 50 suspension. Electrophoresis was performed for 2.5 hours at 45 V/cm as described in section 4.3.5.. Reference peptides were stained for methionine using platinic iodide —o—o— Radioactive profile obtained after incubation without added RNA. -o-o- Radioactive profile obtained after incubation with 14S lens messenger.

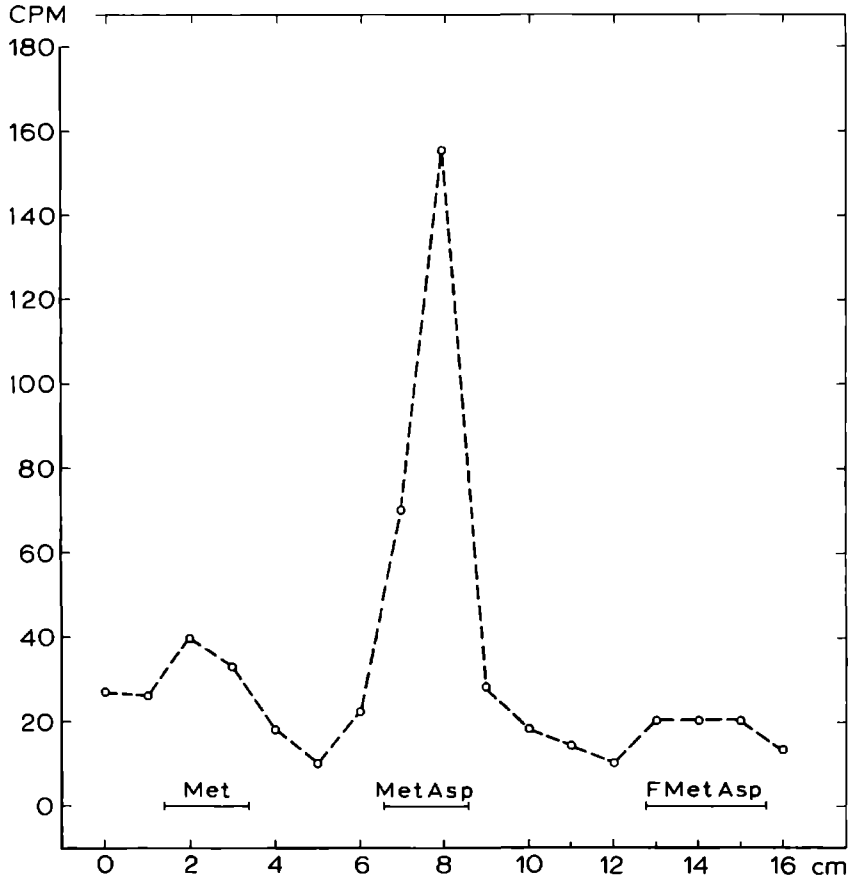


Fig.12. Paper electrophoretic analysis of the deformylated N-terminal dipeptide of polypeptides synthesized under the direction of the 14S lens messenger. Paper segments from the electropherogram (fig.11) corresponding to the formyl-Met-Asp position were removed from the counting vials, washed with fresh toluene and the radioactive material was eluted with 2 ml distilled water. This material was deformylated as described in the text After lyophillization it was subjected to paper electrophoresis at 45 V/cm for one hour Reference peptides were stained for methionine using platinic iodide

ed from lens tissue and formylated with the aid of *E coli* enzyme preparation[152] was used as radioactive precursor. The use of this precursor resulted in an incorporation of about 10^4 cpm per mg protein. Addition of lens messenger inhibited the incorporation up to 60%.

However from the electropherogram in fig.11 it can be seen that in spite

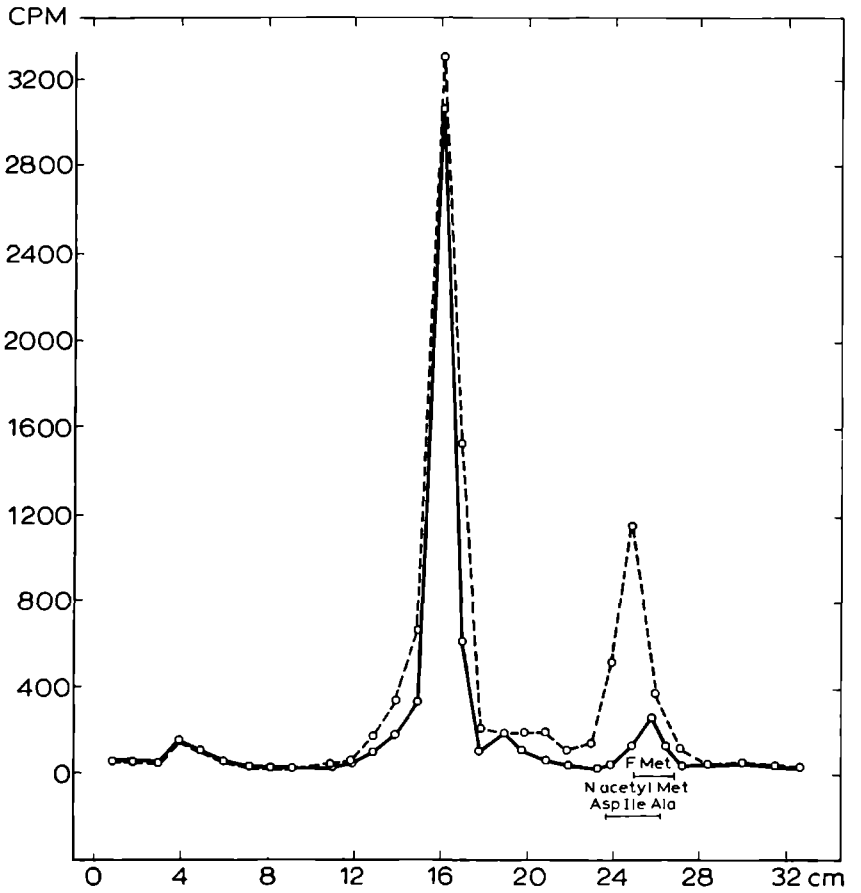


Fig.13 Paper electrophoretic identification of the N-terminal tetrapeptide of polypeptides synthesized under the direction of the 14S lens messenger The peptides, derived from 100 μl incubation mixtures, were digested with subtilisin at 37°C for 6 hours After digestion the peptides were treated with Dowex 50 suspension to remove the unblocked peptides Electrophoresis was performed for 2 hours at 35 V/cm as described in section 4 3 5 Reference peptides were stained for methionine using platinic iodide -o—o- Distribution of radioactivity after incubation without added RNA -o-o- Distribution of radioactivity after incubation in the presence of 14S lens messenger

of this inhibition addition of the 14S lens messenger to the cell-free system resulted in the synthesis of TCA precipitable peptides, which after pronase digestion and Dowex 50 treatment behaved identical to formyl-Met-Asp

To get further evidence for the synthesis of the correct N-terminal peptide

the radioactive material at the position of formyl-Met-Asp was eluted and treated with 0.5 N HCl at 90°C for 30 min [66] Under these conditions the formyl group is removed whereas the acetyl group is not attacked This treatment shifted the radioactivity almost completely to the position of Met-Asp (fig 12).

To complete this N-terminal analysis, the product synthesized in the presence of lens messenger was digested with subtilisin It is known that subtilisin releases the N-terminal tetrapeptide N-acetyl-Met-Asp-Ile-Ala which all four α-crystallin polypeptides have in common As the formylated tetrapeptide was not available we used the acetylated tetrapeptide as reference The electrophoretic mobility of these peptides could be expected to be almost identical Addition of 14S lens messenger to the cell-free system in the presence of formyl-(^{35}S) Met-tRNA$_f^{Met}$ resulted in the formation of a peptide which after subtilisin digestion exhibited the same electrophoretic mobility as acetyl-Met-Asp-Ile-Ala (fig 13)

Elution of the radioactive material at the position of the tetrapeptide and treatment of this material with pronase, resulted in the release of a peptide which exhibited the same electrophoretic mobility as formyl-Met-Asp (fig 14). That only part of the material was shifted to the formyl-Met-Asp position may be due to uncomplete digestion as the pronase treatment in this experiment was carried out for only 2 hours.

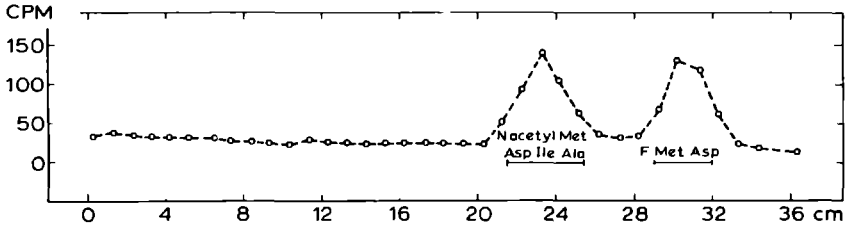


Fig 14 Paper electrophoretic identification of the N-terminal dipeptide derived from the material present at the position of the N-terminal tetrapeptide (fig 13) The radioactive material at the position of the N-terminal tetrapeptide was eluted from the paper (electropherogram depicted in fig 13) and treated with pronase at 37°C for 2 hours After lyophillization of the digested material electrophoresis was performed for 2 hours at 35 V/cm as described in section 4.3.5 Reference peptides were stained for methionine using platinic iodide

From these experiments we may conclude that the addition of the 14S lens messenger to the reticulocyte cell-free system results in the synthesis of

polypeptides with an N-terminal sequence identical to the N-terminal sequence of α-crystallin polypeptide chains

4 4 2 Analysis of 14S RNA Directed Products by Polyacrylamide Gel Electrophoresis

As the N-terminal analysis described in section 4 4 1 does not provide evidence for the completion of the newly synthesized chains and cannot give information about the type of α-crystallin polypeptide chain, we identified the chains further by gel electrophoresis on acidic and basic urea gels and on SDS gels

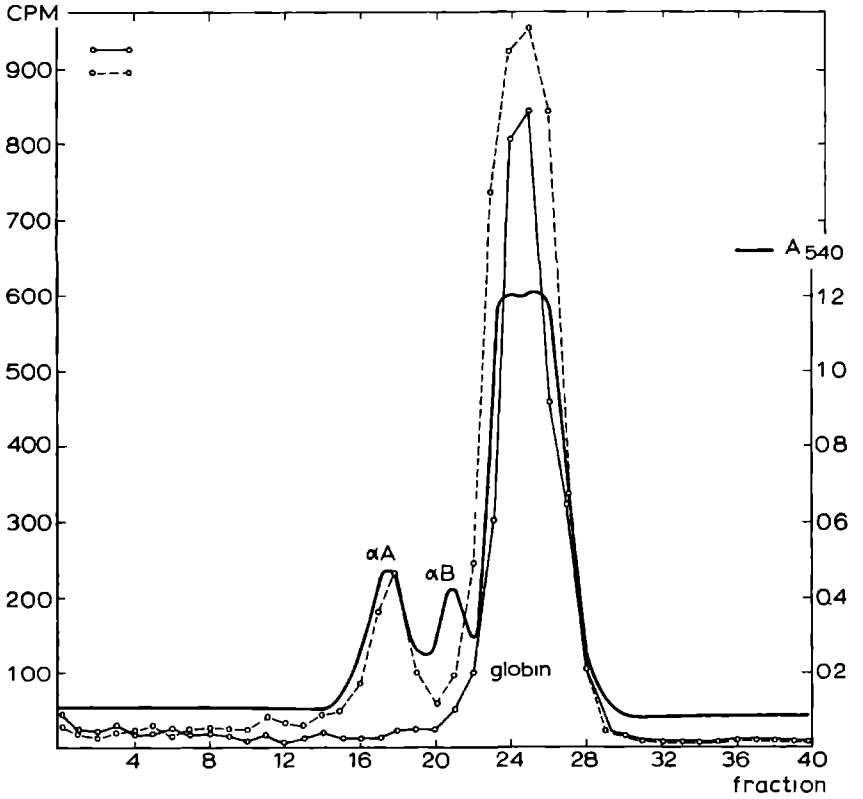


Fig 15 Electrophoretic analysis on acidic urea gels of polypeptides synthesized under the direction of the 14S messenger Gel electrophoresis was performed as described in section 4 3 4 400 μg polypeptides, handled as described for the globin preparation, was applied per gel together with 100 μg carrier α-crystallin —— Absorbance at 540 nm o–o–o Distribution of radioactivity after incubation without added RNA o–o–o Distribution of radioactivity after incubation with 14S lens messenger

Electrophoresis on acidic urea gels of the products made after incubation with 14S lens messenger in the presence of formyl-(^{35}S) Met-tRNA$_f^{Met}$ reveals that the 14S messenger directs the synthesis of an αA polypeptide (fig.15). From this figure it can be seen that apparently no αB chains are synthesized. In addition, electrophoresis on basic urea gels of the polypeptides synthesized in the presence of 14S mRNA indicates that of the αA polypeptides only the αA_2 chain is synthesized (fig.16).

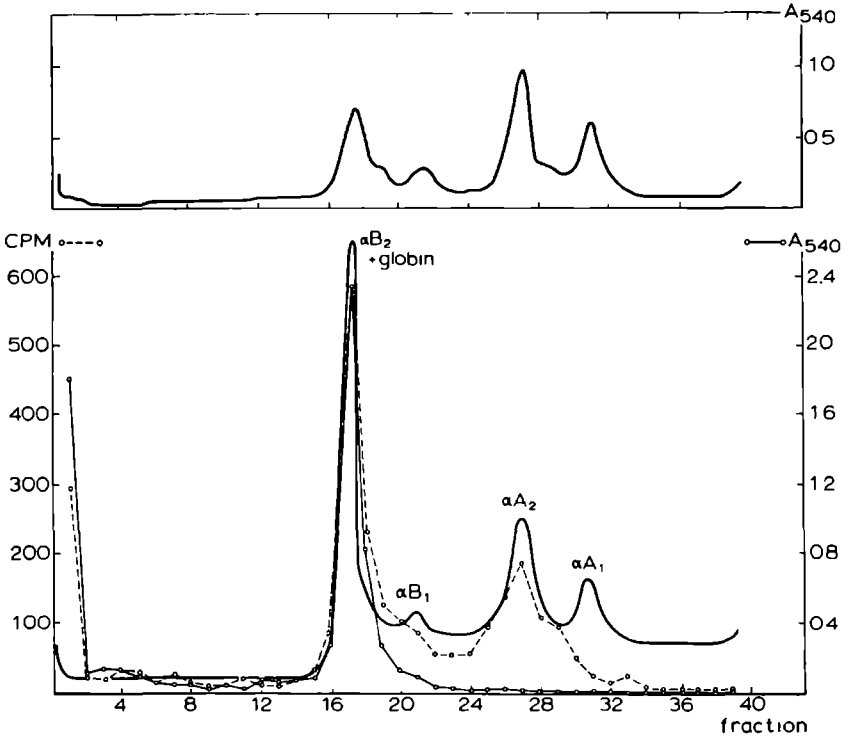


Fig.16. Electrophoretic analysis on basic urea gels of polypeptides synthesized under the direction of the 14S lens messenger. Gel electrophoresis was performed as described in section 4.3.4.. 400 μg polypeptides, handled as described for the globin preparation was applied per gel together with 100 μg carrier α-crystallin.
Upper diagram: Optical density profile of α-crystallin.
Bottom diagram. ——Optical density profile of reticulocyte lysate components + α-crystallin. ~~Distribution of radioactivity after incubation without added RNA. ·-·-· Distribution of radioactivity after incubation with 14S lens messenger.

In order to obtain an estimate of the molecular weight of the product, slices of the alkaline urea gels were subjected to SDS gel electrophoresis. The segment containing the globin and αB chains and the segments corresponding to the αA_2 chain were applied on SDS gels as described in the method section. From the distribution of radioactivity in the gel it could be deduced that the molecular weight of the newly synthesized chains is identical to the molecular weight of the A chains of α-crystallin, which has been demonstrated to be 19,000.[5] Electrophoretic separation of the mixture containing the globin and αB chains revealed that no detectable radioactivity was located in the αB region of the gel. This observation supports the conclusion already drawn from the results obtained from the acidic urea gels; the 14S lens messenger does not direct the synthesis of αB polypeptide chains.

Evaluation of all electrophoretic experiments clearly shows that the addition of 14S lens messenger to a lysate system from reticulocytes results in the synthesis of a polypeptide which is indistinguishable from native A_2 chains of α-crystallin.

4.4.3. Identification of the Two Methionine Containing Tryptic Peptides

In order to have labelled both the N-terminal and internal methionine-containing tryptic peptide we used (^{35}S) Met as radioactive precursor in our incubation mixtures. After incubation a cold acetone precipitation was applied as described in section 4.3.3. and the αA_2 chains were separated from the other polypeptides by electrophoresis on preparative basic urea gels, as described in section 4.3.4.. The polypeptide was aminoethylated, in order to obtain a smaller internal methionine-containing peptide after trypsin digestion, and subjected to paper chromatography (fig.17). Marker peptides derived from a tryptic digest of (^{35}S) methionine labelled calf lens αA_2 chains were run on an adjacent strip of paper. Fig.17 shows that the methionine peptides from reticulocyte lysate derived αA_2 material have the same chromatographic mobility as do the reference peptides, derived from calf lens αA_2-crystallin. The fast moving component shown in fig.17 is the N-terminal peptide and the slow moving component is the internal peptide. The slow moving component is partially resolved into a major and a minor component, the latter representing the oxidized form of this methionine peptide. The two oxidation states of the fast moving component are not resolved in this chromatogram. Fig.17 shows that the internal and N-terminal peptides from reticulocyte derived material are not present in equal amounts: this inequality can also be seen in the (^{35}S) methionine labelled αA_2 reference peptides and is probably the result of incomplete digestion.

We may conclude that the methionine containing peptides derived from αA_2 chains, synthesized in a reticulocyte lysate under the direction of the

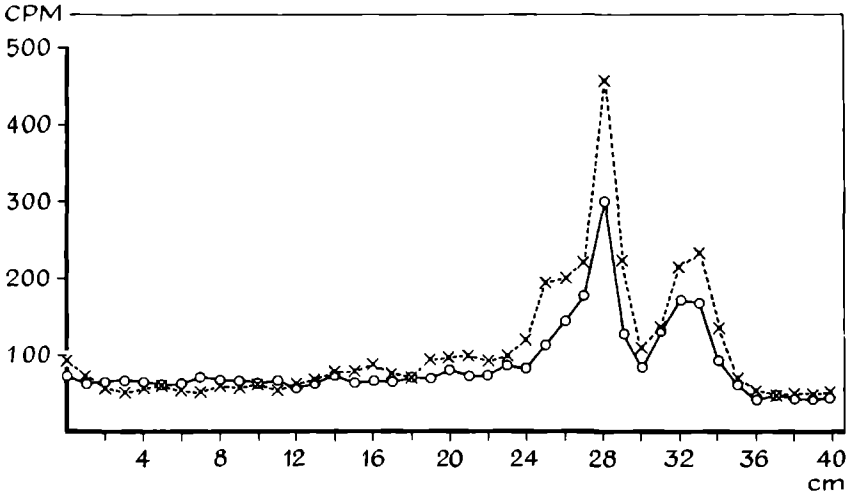


Fig.17. Paper chromatographic analysis of the methionine containing tryptic peptides of polypeptide chains synthesized under the direction of the 14S lens messenger. The peptides were chromatographed as described in section 4.3.5.. -o-o Radioactive distribution obtained with methionine labelled tryptic peptides derived from native αA_2 chains. x-x-x Radioactive distribution obtained with methionine labelled tryptic peptides derived from a reticulocyte lysate programmed with 14S lens messenger

14S lens messenger are indistinguishable, by chromatographic analysis, from the methionine containing peptides derived from native αA_2 chains.

4.4.4. N-Terminal Acetylation

As the analysis in section 4.4.3. suggests that the N-terminal tryptic peptide derived from the reticulocyte system is identical to the N-terminal tryptic peptide of native αA_2 chains, it was interesting to know whether the reticulocyte system is able to acetylate the N-terminus.

As an experimental approach to solve this problem we applied subtilisin and pronase digestion to the αA_2 chains isolated from the reticulocyte system with the aid of basic urea gels. Subtilisin digestion of reticulocyte derived αA_2 released a blocked peptide with the same electrophoretic mobility as N-acetyl-Met-Asp-Ile-Ala, while pronase released a peptide with an electrophoretic mobility identical to N-acetyl-Met-Asp. To exclude the possibility that the blocking group was a formyl group one sample of the pronase digest was treated with 0.5 N HCl at 90°C for 30 min.[66] This treatment did not result in a shift of radioactivity to the position of Met-Asp as described

for the formylated dipeptide (section 4 4 1), in accordance with the stability of the acetyl-Met bond These peptides could not be obtained from incubation mixtures to which no messenger was added

From these experiments we may conclude that the addition of 14S lens messenger to the reticulocyte cell-free system results in the synthesis of A_2 chains of α-crystallin with an acetylated N-terminus This observation will be discussed in terms of the possibility that the protein synthesizing machinery of different tissues possesses an acetylation mechanism which is able to recognize a certain amino acid sequence

4 5. PARTIAL IDENTIFICATION OF THE POLYPEPTIDES MADE UNDER THE DIRECTION OF THE 10S MESSENGER FRACTION FROM LENS

4 5 1 Analysis of the N-Terminal Peptide

As the 14S messenger from lens directs exclusively the synthesis of the A_2 polypeptide of α-crystallin, it is conceivable that the 10S messenger fraction should code for one or more of the other chains of α-crystallin Since all polypeptide chains of α-crystallin possess the same N-terminal sequence, we examined first whether addition of the 10S messenger to the reticulocyte system in the presence of formyl-(^{35}S) Met-$tRNA_f^{Met}$ resulted in the synthesis of polypeptides with the N-terminal sequence N-formyl-Met-Asp-Ile-Ala This analysis, carried out as described in section 4.4 1 resulted in the identification of the same tetrapeptide, indicating that the 10S messenger fraction contains the information for one or more α-crystallin polypeptides Besides the identifiable tetrapeptide some other peptides could be detected on the electropherogram, which have not been further characterized This may indicate that the 10S messenger fraction contains also messengers coding for polypeptides differing from α-crystallin chains

4 5 2. Analysis of the 10S RNA Directed Products by Polyacrylamide Gel Electrophoresis

Since the N-terminal analysis described in section 4 5 1 does not prove the completion of the chains and cannot provide information concerning the type of polypeptide synthesized we further identified the chains with the aid of SDS gels As the 10S messenger fraction seemed to contain different messengers we divided the 10S after zonal centrifugation into small fractions This fractionation is visualized in fig.18

We identified the products made under the direction of these different messenger fractions in a reticulocyte lysate with (^{35}S) methionine as radioactive

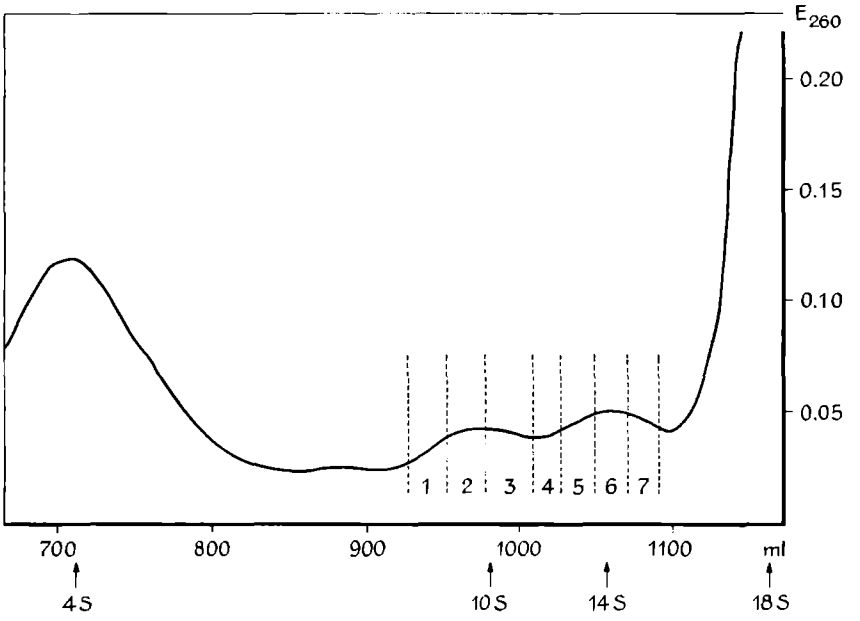


Fig.18. Zonal centrifugation profile of polyribosomal RNA from calf lens. Only part of the profile is visualized. Centrifugation was performed in a B XXIX zonal rotor of IEC for 25 hours. Further conditions were as described in section 2.3.2. (gradient 3).

precursor. From the autoradiographs of the SDS gels it can be deduced that there is a significant difference in messenger information between the subsequent messenger fractions (fig.19). While fraction 2 seems to direct mainly the synthesis of αB chains, fraction 3 and 4 contain the information for other polypeptide chains, probably β- or γ-crystallins. On the other hand, the 14S messenger fractions give rise to the synthesis of αA_2 chains only.

In the first three gels there is a clear relationship between the molecular weight of the newly synthesized protein and the sedimentation value of the messenger fraction: a messenger with a higher S value synthesizes a protein with a higher molecular weight. However in the last three gels this relation does not hold any more since the molecular weight of the polypeptide synthesized under the direction of the 14S messenger is significantly lower as compared with the molecular weights of the polypeptides synthesized under the direction of the 10S messenger fractions.

The significance of this observation will be discussed in more detail in sectio 4.6.

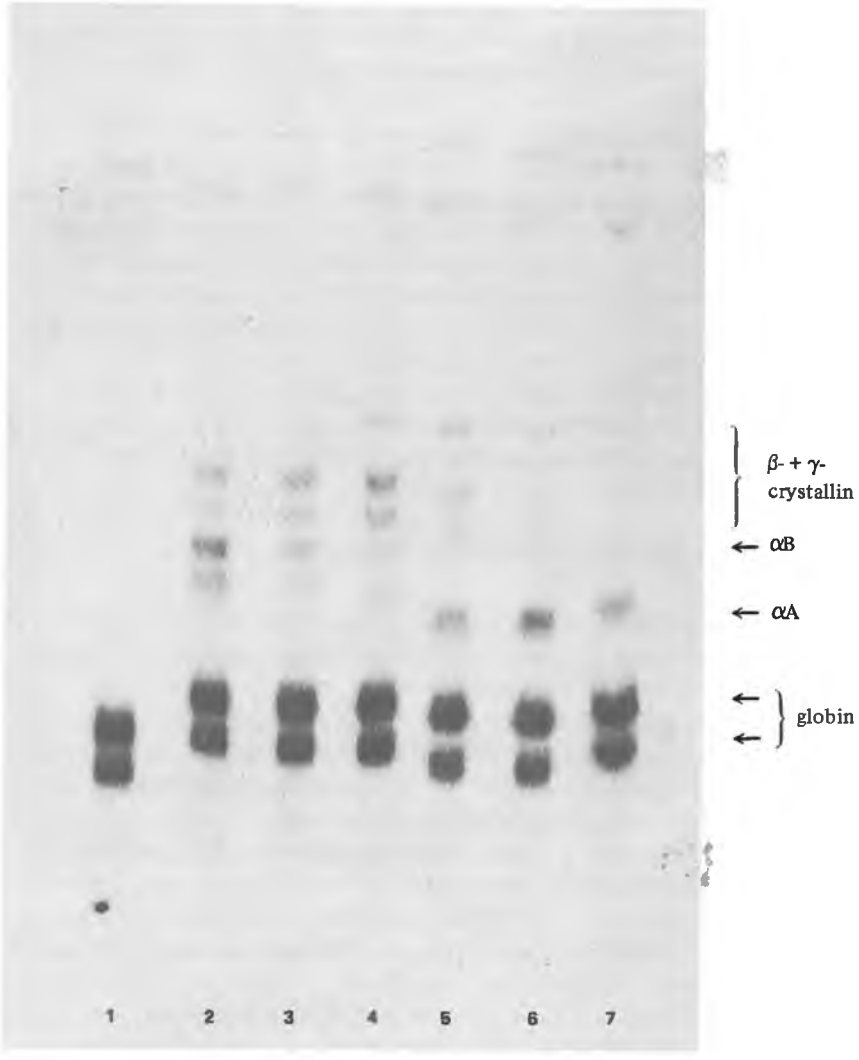


Fig.19. Autoradiographs of the SDS gels containing the products made under the direction of the messenger fractions 2-7, visualized in fig.18. SDS gel electrophoresis was carried out as described in section 4.3.4.. Per gel 50 μg protein was applied. The dried gels were autoradiographed during three weeks.

From the autoradiographs one may draw the erroneous conclusion that the messenger activity of the 10S fraction is distributed almost equally over

the three different polypeptides. It will be shown in chapter 5 that the amount of polypeptides other than αB is exaggerated as the methionine content of these polypeptides appeared to be significantly higher.

4.5.3. N-Terminal Acetylation

We identified the N-terminal sequence of the polypeptides made under the direction of messenger fraction 2 (see fig.18) as this fraction appeared to synthesize mainly αB chains (see fig.19). Subtilisin and pronase digestion on the total incubation mixture followed by Dowex 50 treatment and paper electrophoresis revealed that addition of the 10S messenger fraction 2 resulted in the synthesis of polypeptides with an N-terminal sequence identical to N-acetyl-Met-Asp-Ile-Ala and N-acetyl-Met-Asp respectively. Therefore we may conclude that the addition of 10S messenger to the reticulocyte system results in the synthesis of at least one of the B chains of α-crystallin and that the reticulocyte lysate is able to acetylate the N-terminus.

4.6. DISCUSSION

The messenger function of the different RNA fractions from calf lens is clearly demonstrated by peptide analysis and gel electrophoresis. From these results we may conclude the following:

The reticulocyte cell-free system is able to recognize and translate faithfully a messenger originating from a highly specialized tissue as the lens without requiring additional lens specific protein factors. This in contrast with results obtained with purified cell-free systems derived from terminally differentiated tissues[56,130] but in accordance with results obtained with crude cell-free systems[101,102,111,132,149] and the *in vivo* system of oocytes from Xenopus laevis.[50,90] Although the lens mRNAs are translated about 30 times less efficient than the endogenous globin mRNA, the results indicate that tissue specific recognition factors, if they are involved, are not obligatory. The most obvious explanation for the apparently contradictionary reports in literature would be: the existence of different types of initiation factors which exhibit varying affinities towards different messengers and the occurrence of which is not restricted to one tissue or species. It is feasible that there are parameters of a more general nature, determining the occurrence of one type of initiation factor in a certain tissue.

It is quite remarkable that the 14S lens messenger, with a molecular weight of about 360,000 (section 3.2.3.) is required to direct the synthesis of a polypeptide of 19,000 daltons only, while the 10S fractions with an average

molecular weight of about 260,000 (section 3 2 3) direct the synthesis of polypeptides with significantly higher molecular weights This observation is strongly supported by the demonstration that in the 10S fractions a relation exists between the sedimentation value of the messenger and the molecular weight of the polypeptide synthesized under its direction, while this relation is not valid for the 14S messenger Three possibilities may be considered

1 The 14S messenger does contain untranslated regions of considerable length The occurrence of large poly A tracks as reported for HeLa messenger RNA,[38] mouse ascites messenger RNA[95] and globin messenger RNA,[25,97] however is not very likely as the AMP content of the 14S messenger seems to be rather low
2 The 14S messenger may be bicistronic, either with a repeating sequence for αA_2 or a sequence for αA_2 and (an)other unknown polypeptide(s)
3 The initial product may be a longer precursor molecule which is proteolitically trimmed to yield αA_2-crystallin Such post-translational modification has a precedent in the processing of virus coded proteins in picorna virus-infected cells [7]

Which of these alternatives is correct has still to be elucidated

As an acetylated methionine occurs at the N-terminus of all α-crystallin polypeptide chains in the lens as well as in the reticulocyte system after incubation in the presence of lens messengers, the following initiation mechanisms may be considered

1 Lens messengers require initiation with acetyl-Met-tRNA$_f^{Met}$
2 The initiation takes place with Met-tRNA$_f^{Met}$ and cleavage of the methionine is prevented by the nature of the adjacent amino acid residue(s), which might determine the specificity of the splitting enzyme Such a specificity has been reported for ribosome bound leucine aminopeptidase in *E coli* [107] An alternative explanation might be that a specific acetylation mechanism prevents that the N-terminal methionine, derived from Met-tRNA$_f^{Met}$ is split off
3 The lens messengers coding for α-crystallin polypeptide chains code for a starting sequence Met-Met-Asp in which the first methionine, derived from Met-tRNA$_f^{Met}$ is split off in the usual manner and the second, derived from Met-tRNA$_m^{Met}$ is acetylated in a later phase

The first possibility is unlikely No acetylated Met-tRNA$_f^{Met}$ could be detected in the lens[151] and reticulocyte system [69]

The third possibility is excluded by the experiments with formyl Met-tRNA$_f^{Met}$ as formyl-Met-Asp was detected after translation of lens messengers in the reticulocyte system

Therefore the N-terminal methionine in α-crystallin chains is derived from

Met-tRNA$_f^{Met}$. The most obvious explanation for the acetylation of α-crystallin chains in the reticulocyte cell-free system is that the protein synthesizing machinery of different tissues, from different species, possesses an acetylation mechanism which is able to recognize and acetylate a certain amino acid sequence.

One may speculate that all eukaryotic cells possess an acetylation mechanism, ribosome bound or free in the cytoplasm of this general nature.

The observation that neither the 14S messenger nor the 10S messengers seem to contain the information for the A_1 chain of α-crystallin is in accordance with the postulation of Palmer and Papaconstantinou, that the occurrence of αA_1 chains is not the result of direct genetic expression. Although not very likely it cannot be excluded that a messenger coding for αA_1 exists. This hypothetical messenger could be lost during the isolation procedure or its translation might be blocked.

TRANSLATION OF LENS MESSENGERS IN THE CELL-FREE SYSTEM DERIVED FROM KREBS II ASCITES CELLS *

5 1 INTRODUCTION

A similar introduction as given in chapter 4 could be repeated here The data reported in the present chapter confirm and extend the results reviewed in chapter 4 Moreover additional evidence is provided that no tissue specific initiation factors are required for lens mesenger translation

5.2. METHODS

5 2 1. Growth of Ascites Cells and Preparation of the S-30 Lysate

Growth of ascites cells, and preparation of the S-30 lysate was carried out as described by M B Mathews and A Korner

5 2 2 Incubation Conditions

The incubation mixture contained per 50 μl 20 μl preincubated ascites S-30, 2.5 mM magnesium acetate, 100 mM KCl, 25 mM Tris-HCl, pH 7 5 6 mM 2-mercaptoethanol, 1 mM ATP, 0 1 mM GTP, 5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, (^{14}C) amino acid mixture 5 μCi/ml (54 mCi/matom, Radiochemical Centre Amersham), supplemented with the six missing amino acids (asparagine, cysteine, glutamine, histidine, methionine and tryptophan) at 20 μM each or (^{35}S) methionine 10 μM. 10 Ci/mmol. plus the remaining nineteen unlabelled amino acids, 20 μM each Lens messenger was added at 60 μg/ml After 60 min at 37°C reactions were terminated by incubation for 15 min at 37°C with pancreatic RNase (20 μg/ml) and 10 mM EDTA. This mixture was either used directly for SDS gel analysis or precipitated with 5% TCA and washed 5% TCA twice, ethanol, ethanol ether (1 1 v/v) and ether, and used for gel electrophoresis on urea gels

5 2 3 Polyacrylamide Gel Electrophoresis

Gel electrophoresis on acidic and basic urea gels was carried out as described in section 4.3 4.

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* This work was carried out in collaboration with Dr M B Mathews of the M R C Laboratory of Molecular Biology, Hills Road, Cambridge, England

For SDS gel electrophoresis of the products of the cell-free incubation, SDS and 2-mercaptoethanol were added to 1% and 2% respectively, together with 5-10 μg of unlabelled α- or β-crystallin markers, to the incubation mixture and the entire solution was heated to 100°C for 2 min

Gels contained 15% acrylamide, 0 4% methylene-bisacrylamide, 0 1% SDS, they were 9 cm long A stacking gel was used, and the gels were prepared and run as described by Laemmli, except that the ionic strength of the running buffer was doubled The gels were stained and destained according to Weber and Osborn and longitudinal slices were dried before autoradiography

5 3. IDENTIFICATION OF THE POLYPEPTIDES MADE UNDER THE DIRECTION OF THE 10S AND 14S LENS MESSENGERS IN THE ASCITES CELL-FREE SYSTEM

In contrast to the non-preincubated system, derived from reticulocytes, in which total incorporation is reduced by the addition of extra messenger, the ascites S-30 evinces a stimulation (fig 20) when supplemented with either 10S or 14S messengers As with globin messenger the two lens messenger fractions are translated about once on average

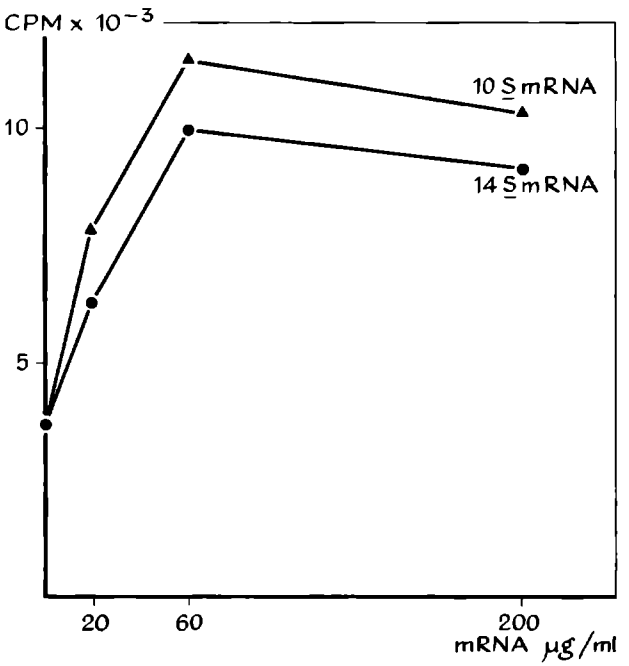

Fig 20 Stimulation of protein synthesis in the ascites cell-free system by lens messengers

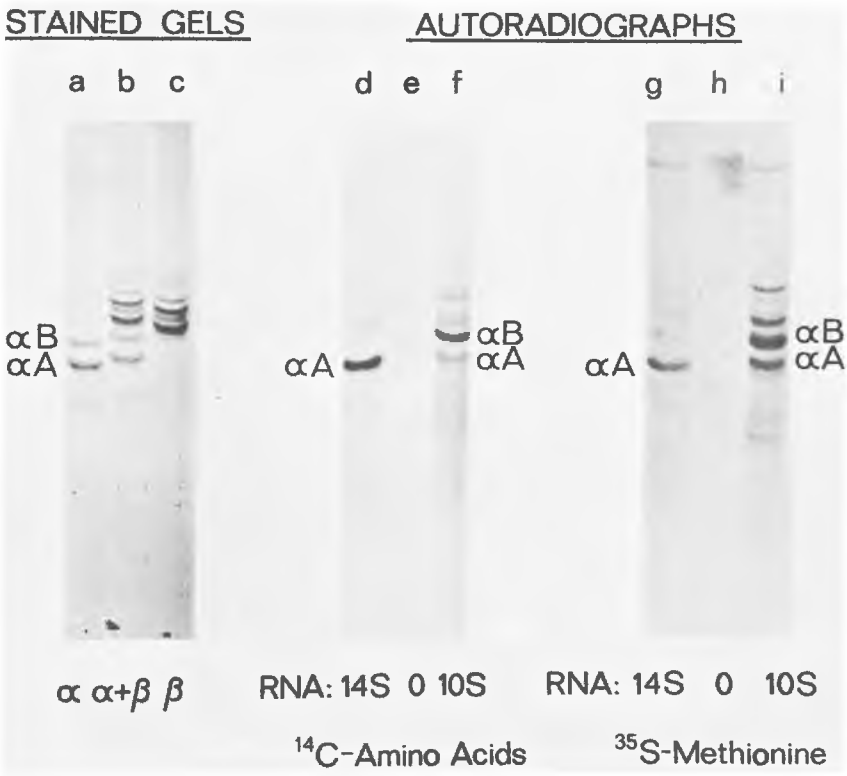

Fig.21. Electrophoretic analysis on SDS gels of the products made in the ascites cell-free system under the direction of lens messengers. Autoradiography was carried out for 4 days.

The products have been identified both on SDS gels which separate on the base of molecular weight and on acidic and basic urea gels in the absence of SDS which separate on the base of charge.

The products of cell-free protein synthesis were mixed separately with unlabelled α- or β-crystallin and electrophoresed on SDS gels, which were subsequently stained for protein, sliced longitudinally and prepared for autoradiography. The polypeptide chains of the added crystallin markers were clearly distinguishable and identifiable in the stained gels against the background of the ascites cell proteins. The autoradiographs of the endogenous and messenger-stimulated ascites cell products are shown in fig.21. It is clear that the endogenous ascites products, labelled in the absence of added messenger did not reveal any radioactivity in the region between 15,000 and

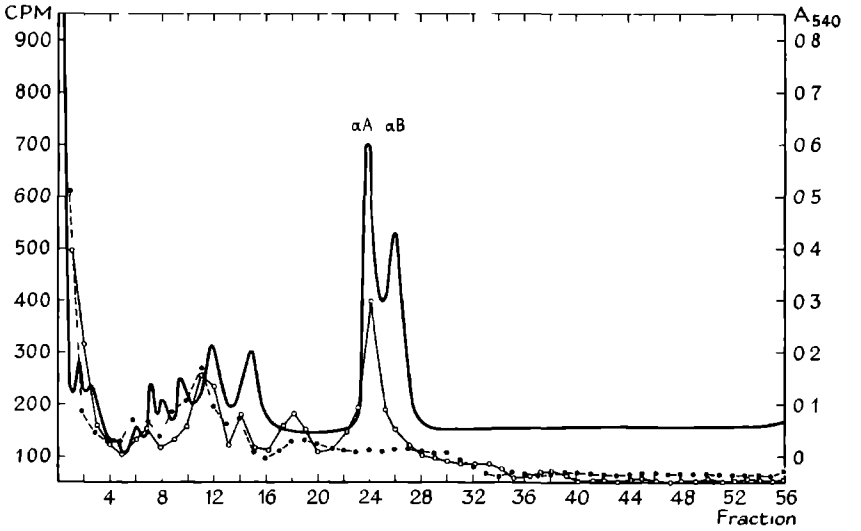

Fig 22 Electrophoretic analysis on acidic urea gels of the products made in the ascites cell-free system under the direction of the 14S lens messenger Gel electrophoresis was performed as described in section 4 3 4 Per gel the material of a 10 μl incubation was applied ——Optical density profile of ascites components + α-crystallin -·-·-·Radioactive distribution after incubation in the absence of added RNA —— Radioactive distribution after incubation in the presence of 14S lens messenger

30,000 daltons, when either (^{35}S) methionine (fig 21h) or (^{14}C) amino acid mixture (fig 21e) was used as label

The 14S messenger gives rise to a single band which coelectrophoresed exactly with the A chains of α-crystallin, when either (^{35}S) methionine (fig 21g) or (^{14}C) amino acid mixture (fig 21d) was used as label There was little sign of contamination with other radioactive products

The main product of the 10S messenger fraction was the B chain of α-crystallin, with lesser amounts of material which migrated with the acidic chains of α-crystallin and also some β- (or possibly γ-) chains (fig 21f) The apparent proportion of β- and γ-crystallin chains in the 10S messenger product is exaggerated when (^{35}S) methionine is used as label (fig 21i), presumably because of their higher content of this amino acid [13] Furthermore comparison of fig 21d and fig 21g shows that this label leads to an underestimate of the amount of αA synthesized under the direction of the 14S messenger relative to the amount of αB in the 10S product This is unexpected as the methionine content of αA- and αB-crystallins are similar [141]

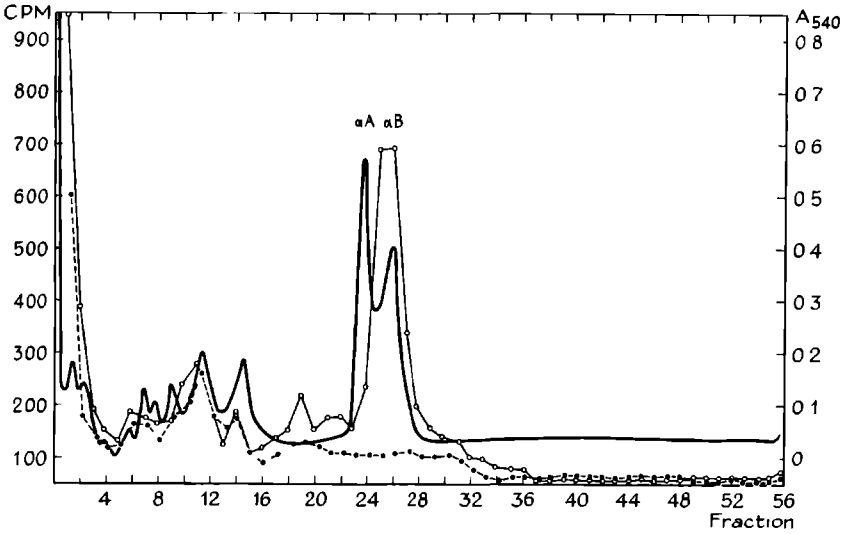

Fig 23 Electrophoretic analysis on acidic urea gels of the products made in the ascites cell-free system under the direction of the 10S lens messenger fraction Gel electrophoresis was performed as described in section 4 3 4. Per gel the material of a 10 µl incubation was applied —— Optical density profile of ascites components + α-crystallin ····Radioactive distribution after incubation in the absence of added RNA ~~~ Radioactive distribution after incubation in the presence of 10S messenger fraction

Gels run at pH 3.0 in urea, which separate the A and B chains of α-crystallin[142] support these conclusions but do not allow further identification of the cell-free products (fig.22 and fig 23)

Urea gels run at pH 8.9 provide further identification of the cell-free products since the αA- and αB-fraction are resolved as mentioned earlier The (^{35}S) methionine labelled cell-free products give the profiles shown in fig 24. The 14S messenger gives rise almost exclusively to αA_2 which may be a precursor of αA_1.[121] The 10S messenger products seem to contain both αB_2 and αB_1, though univoqual identifications cannot be made because some β-crystallin chains also migrate in this position.

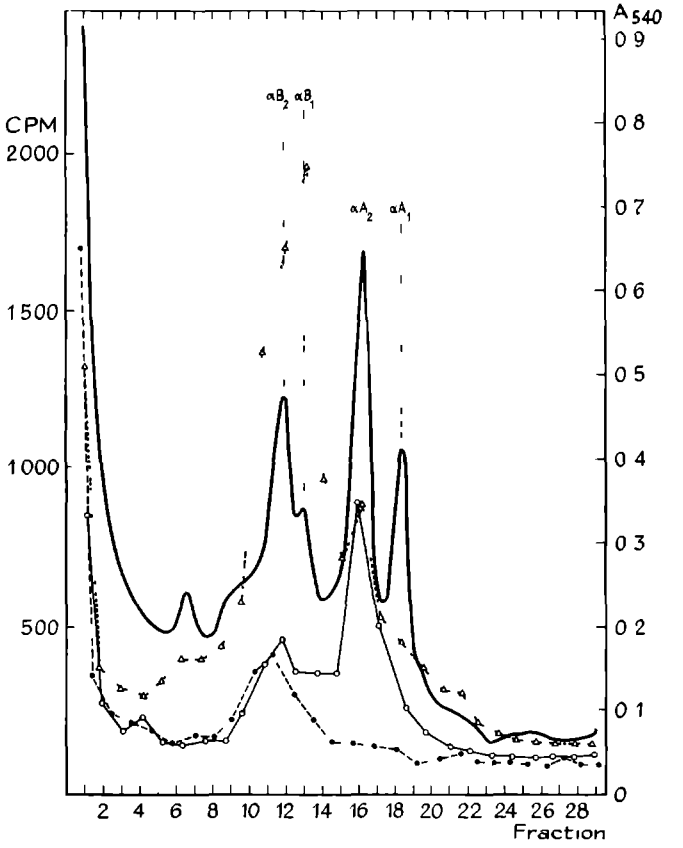

Fig 24 Electrophoretic analysis on basic urea gels of the products made in the ascites cell-free system under the direction of the 10S and 14S lens messengers Gel electrophoresis was performed as described in section 4 3 4 Per gel the material of a 10 μl incubation was applied. ——Optical density profile of ascites components + α-crystallin • • •Distribution of radioactivity after incubation without added RNA ⊷ Distribution of radioactivity after incubation in the presence of 14S lens messenger ∘ ∘ ∘ Distribution of radioactivity after incubation in the presence of 10S lens messengers

5.4 DISCUSSION

Our results indicate that the two lens messenger fractions are active in the Krebs II ascites cell-free system and direct the synthesis of at least two identifiable lens polypeptide chains Again it is surprising that the messenger

coding for αA_2 (MW = 19,000) sediments as rapidly as 14S, in contrast to the messengers coding for globin (MW 16,000) and crystallin αB (MW 22,000) which sediment at 9S[26,101] and 10S[9] respectively The possible explanations for this phenomenon have already been discussed in the foregoing chapter

The lens messengers are translated in the Krebs II ascites cell-free system with no requirements for additional lens components Similarly lens (chapter 4) myeloma[149] and ovalbumin[132] messengers have been translated in the reticulocyte system, and globin messenger in Xenopus laevis [90] and in cell-free extracts from Krebs II[111] and Landschutz ascites cells and rat and mouse liver [108] These results fortify the conclusion drawn in section 4 6 that neither tissue specific nor species specific initiation factors are obligatory, at least in these instances.

The experiments with the ascites system did not sustain the speculation done in section 4 6. that all eukaryotic systems contain an acetylation mechanism which is able to acetylate a certain amino acid sequence we were not able to identify the blocked tetrapeptide This may be due to the fact that the S-30 lysate from ascites cells is preincubated and filtrated through Sephadex G-25 in order to remove all low molecular weight material During the preincubation the endogenous acetyl-CoA may be consumed or it may be lost during the filtration step. Therefore the occurrence of the acetylation mechanism in the Krebs II ascites system cannot be excluded by these experiments.

Finally, the present work and the work with globin mRNA,[111] show that the ascites system combined with SDS gel electrophoresis gives a rapid and sensitive assay for messenger activity

TRANSLATION OF 14S LENS MESSENGER IN THE LIVING OOCYTE OF XENOPUS LAEVIS *

6.1. INTRODUCTION

Several interesting questions in developmental biology can be studied by combining messenger from one kind of cell with the translational apparatus of another cell type. Examples of such cross systems have been described in the foregoing chapters.

When rabbit reticulocyte 9S mRNA is injected into frog oocytes, haemoglobin is synthesized, showing the absence of any requirement for tissue specific factors and the presence, within the oocyte, of non-specific translational systems.[90]

In this chapter we shall describe the translation of the 14S lens messenger in frog oocytes.

6.2. METHODS

6.2.1. Handling of Oocytes

The injection procedure and culture medium were as described by Lane, Marbaix and Gurdon, except that batches of 40 oocytes were incubated at 21°C for 20 hours in a medium containing (^{35}S) methionine (26 Ci/mmol at 0.5 mCi/ml). 14S mRNA was dissolved in injection medium (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl, pH 7.6) at a concentration of 2 mg/ml. Per oocyte 50 mμl was injected.

6.2.2. Homogenization of Oocytes

Thawed samples of oocytes were homogenized in 10 μl per oocyte of a medium containing 0.05 M glycine, 0.05 M Tris, 0.08 M KCl, 0.05 mM methionine and unlabelled calf lens α-crystallin (100 μg/ml). The final pH was 8.9.. The homogenate was centrifuged at 3500 g for 15 min at 4°C. To the supernatant 1/25 volume of 0.2 M EDTA and 1/10 volume 0 2 mg/ml pancreatic ribonuclease were added, and after incubation at 37°C for 15

* This work was carried out in collaboration with Dr C D Lane, Department of Zoology, University of Oxford, Oxford, England

min, 1/5 volume of 50% TCA was added: The precipitated protein was washed four times with 5% TCA, once with ethanol, once with ethanol-ether (1:1 v/v) and once with ether. The material, after drying at room temperature, was subjected to electrophoretic analysis.

6.2.3. Polyacrylamide Gel Electrophoresis

Samples which contained about 75,000 cpm of (^{35}S) methionine labelled material, were mixed with 25 μg of marker α-crystallin, and were electrophoresed in acidic and basic urea gels as described in section 4.3.4..

For SDS gel electrophoresis, carried out as described in section 4.3.4., no marker α-crystallin was added, as enough crystallin was present after the addition of carrier α-crystallin in the homogenization procedure.

6.2.4. Peptide Analysis

Peptide analyses were carried out as described in section 4.3.5..

6.3. IDENTIFICATION OF THE POLYPEPTIDE MADE UNDER THE DIRECTION OF THE 14S LENS MESSENGER IN THE LIVING OOCYTE

Oocytes were injected with calf lens 14S messenger dissolved in injection medium and were frozen after 20 hours' incubation in (^{35}S) methionine. Control oocytes were treated similarly, except that the 14S messenger was omitted from the injection mixture. This incubation resulted in an incorporation of about $1.5 \cdot 10^5$ cpm/oocyte. Newly synthesized proteins from both batches of oocytes were then analysed for their content of crystallin-like material.

6.3.1. Analysis of the 14S RNA Directed Products by Polyacrylamide Gel Electrophoresis

The radioactive oocyte proteins were first analysed by SDS gel electrophoresis, a technique which is known to resolve α-crystallin into two components, αA and αB, having molecular weights of 19,000 and 22,000 respectively.[5]

Figure 25a shows that the presence of 14S messenger in the injection mixture is associated with the formation of radioactive molecules that coelectrophorese with the αA component of added marker α-crystallin. Scanning of the autoradiogram shows that when material from 14S RNA injected oocytes is run on SDS gels at least 15% of the total radioactivity present in the gel can be found in the αA band; but only 2% of the total radioactivity can be found in the αB band. Moreover less than 2% of the

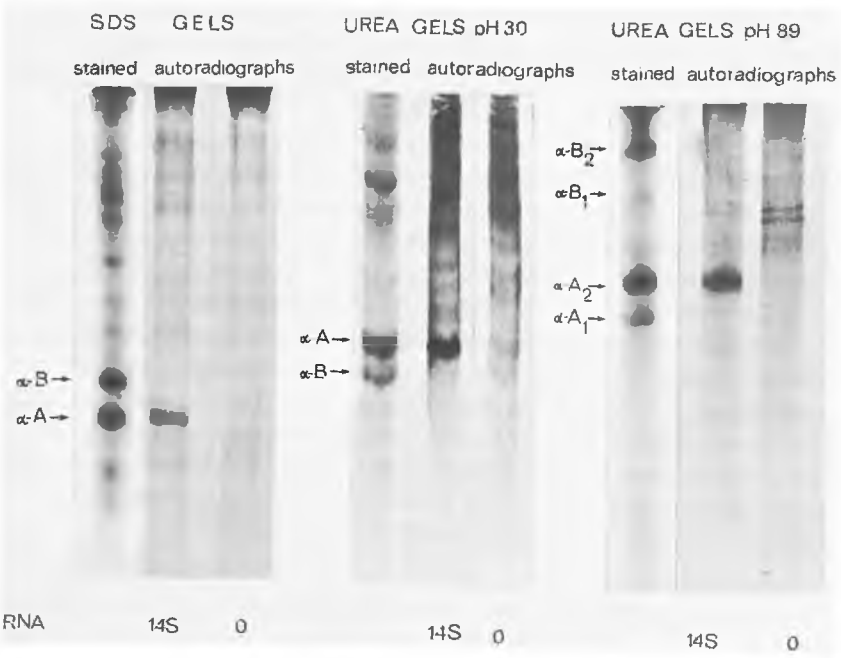

Fig.25. Electrophoretic analysis on polyacrylamide gels of the polypeptides synthesized in the living oocyte under the direction of the 14S lens messenger. Electrophoresis was performed as described in section 6.2.3.. Autoradiography was performed for 16 hours.

radioactivity from control oocytes coelectrophorese with marker αA-crystallin chains. Less than 2% of the total radioactivity from control samples move with marker αB chains.

Acidic urea gels also separate the acidic αA-component from the basic αB-component.[142] Figure 25b confirms that the presence of 14S messenger in the injection mixture is associated with the formation of oocyte-derived material coelectrophoresing with the A-component of marker α-crystallin.

Basic urea gels resolve α-crystallin into its four constituent polypeptide chains, αA_1, αA_2, αB_1 and αB_2.[140] (^{35}S) methionine labelled material from 14S RNA injected oocytes when analysed on such a basic urea gel was shown to contain labelled material, amounting to 20% of the total radioactivity, that coelectrophoresed with the A_2 chain of marker crystallin. No radioactive material preferentially migrates to the αA_1, αB_1 or αB_2 regions of the gel. Less than 4% of the total radioactivity from control oocytes migrate to the αA_2 region of the gel.

If 14S lens messenger injected oocytes and controls are labelled with a mixture of tritiated amino acids (lysine, leucine and histidine) the three types of gel analysis described again reveal labelled αA_2-crystallin chains in messenger injected but not in control oocytes

6.3.2. Identification of the two Methionine Containing Tryptic Peptides

αA_2 chains isolated from basic urea gels were digested with trypsin, and the resulting peptides were subjected to paper chromatography as described in section 4 3.5.

Fig 26 shows that the methionine peptides from oocyte derived αA_2 material have the same chromatographic mobility as do the reference peptides derived from calf lens αA_2 material, while methionine peptides from control oocytes did not reveal this mobility. The fast moving component shown in fig 26 is the N-terminal peptide, and the slow moving component is the internal peptide. The explanation for the shoulder in the slow moving peptide and the inequal amounts of the internal and N-terminal peptide has already been given in section 4.4.3..

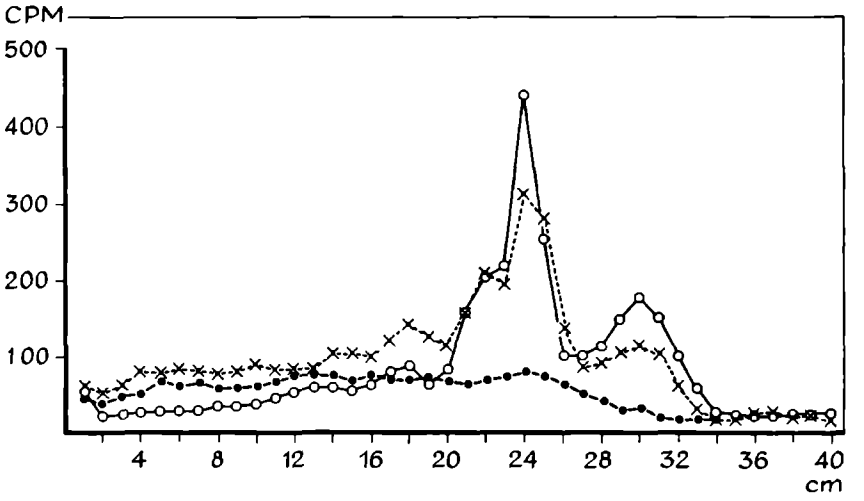

Fig 26 Paper chromatographic analysis of the methionine containing tryptic peptides of polypeptide chains synthesized in the oocyte under the direction of the 14S lens messenger The peptides were chromatographed as described in section 4 3 5 -o-o-o- Distribution of radioactivity obtained with methionine labelled tryptic peptides derived from native αA_2 chains ×-×-× Distribution of radioactivity obtained with methionine labelled tryptic peptides from 14S injected oocytes -•-•-• Distribution of radioactivity obtained with methionine labelled tryptic peptides derived from control oocytes

6.3.3. N-Terminal Acetylation

It has been mentioned earlier that the N-terminal methionine of αA_2 chains from calf lens is acetylated (section 4.2.). The similarity in chromatographic behaviour between the N-terminal peptides of oocyte-derived and lens-derived αA_2 chains suggests, but does not prove, that the N-terminal methionine residue is also acetylated in chains made in the oocyte. The N-terminal sequence of all α-crystallin polypeptides is N-acetyl-Met-Asp-Ile-Ala, and subtilisin releases a peptide of this sequence.[62] Pronase releases the dipeptide N-acetyl-Met-Asp. Fig.27 shows the result of electrophoresing the products of subtilisin digestion of (^{35}S) methionine labelled αA_2 chains from oocytes; fig.28 shows the result obtained from a pronase digestion.

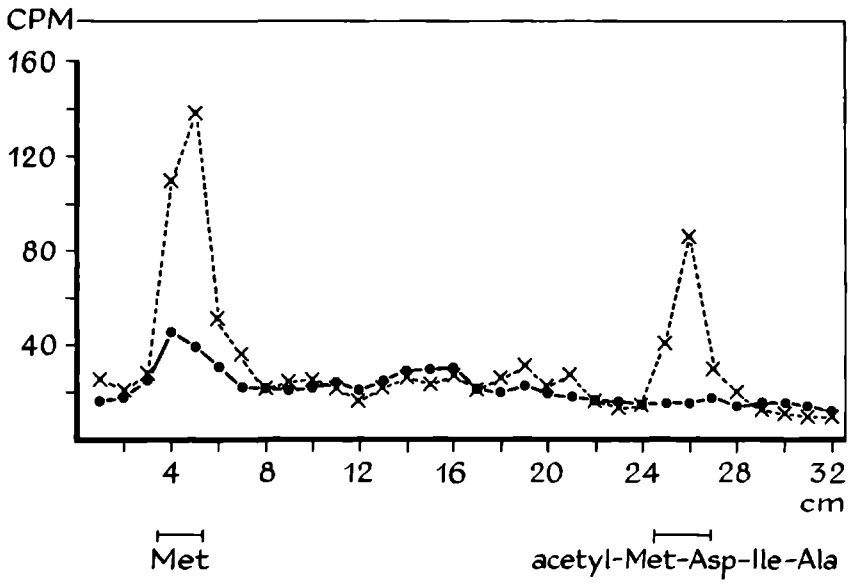

Fig.27. Paper electrophoretic analysis of the N-terminal tetrapeptide from oocyte derived αA_2 chains. Electrophoresis was performed as described in section 4.3.5.. αA_2 chains were isolated with the aid of basic urea gels. ×–× Distribution of radioactivity obtained after incubation in the presence of 14S lens messenger. •–• Distribution of radioactivity obtained after incubation without added RNA.

The results demonstrate that the N-terminal peptide from oocyte derived αA_2 chains is blocked: for in the case of the pronase digestion, the electrophoretic system has been shown to be capable of resolving the free and

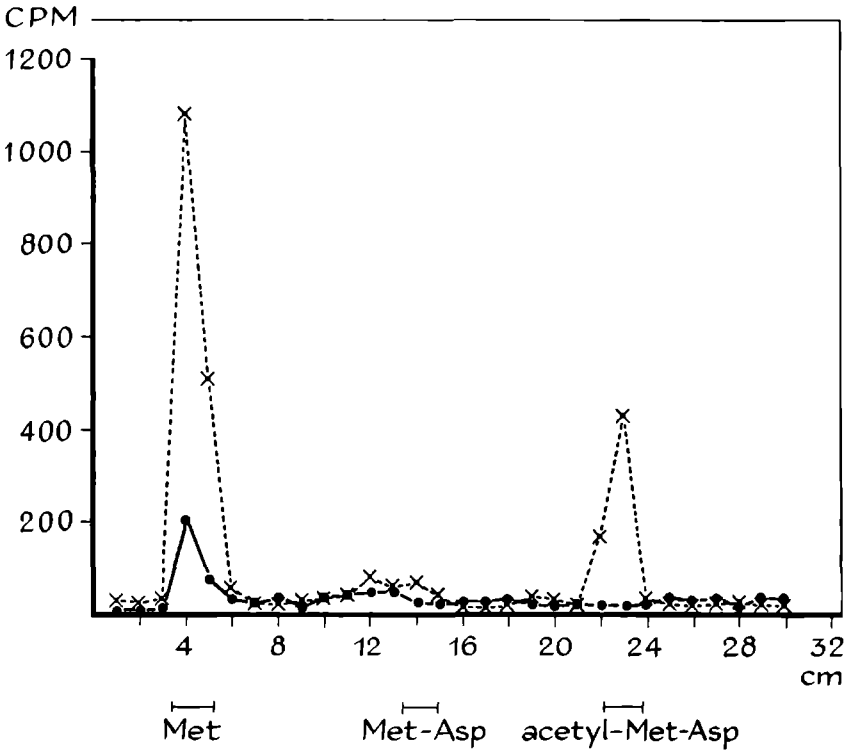

Fig.28. Paper electrophoretic analysis of the N-terminal dipeptide from oocyte derived αA_2 chains. Electrophoresis was performed as described in section 4.3.5.. αA_2 chains were isolated with the aid of basic urea gels ×-×-× Distribution of radioactivity obtained after incubation in the presence of 14S lens messenger. -•-•-Distribution of radioactivity obtained after incubation without added RNA.

blocked N-terminal peptides. To exclude the possibility that the blocking agent is a formyl group, one sample of the N-terminal dipeptide was heated with 0.5 N HCl for 30 min at 90°C.[66] There was no shift of radioactivity to the Met-Asp region of the electropherogram One may conclude therefore that frog oocytes programmed with 14S mRNA from calf lens synthesize material that is extremely similar, if not identical to A_2 chains of calf lens α-crystallin, even to the extent that the oocyte derived αA_2 chains are acetylated.

Our results indicate that the 14S messenger from calf lens codes for αA_2-crystallin chains, in exact accordance with information obtained using cell-free systems (chapter 3 and 4) However the experiments described do not *prove* that 14S RNA injected oocytes synthesize calf αA_2-crystallin chains it is conceivable that the 14S RNA elicits the synthesis of frog crystallin chains However, this possibility is virtually ruled out by data obtained for the analogous situation of oocytes injected with rabbit reticulocyte 9S RNA [89,90,91]

The results also contribute to the question of the species and cell-type specificities, and requirements, of the translation process Since calf lens crystallin mRNA is successfully translated in frog oocytes one can conclude that the components required to translate calf lens αA_2-crystallin messenger are present in cells as unrelated as calf lens cortex cells and frog oocytes Thus, if messenger-specific components are required for the translation of the crystallin messenger, then such components are present and available in frog oocytes If it is assumed that such factors do exist in oocytes, then it is clear that their presence cannot be the only phenomenon determining the appearance of cell-type specific proteins during cell differentiation The results strongly suggest, but do not prove that the translational machinery of frog oocytes is not cell-type specific

These observations, showing that no additional factors are required for the translation of exogenous messengers, are consistent with other experiments performed using whole oocytes,[90] and with results obtained from a variety of crude cell-free systems[101,132,149] (chapter 4 and 5)

Evidence for tissue specificity has only been found using purified cell-free systems derived from terminally differentiated tissues [56,130] These experimental results are not necessarily inconsistent, for it is possible that tissue specificity is masked in crude cell-free systems, and that oocytes do not have such restrictive translational requirements as do cells of terminally differentiated tissues

The chains of native α-crystallin are N-terminally acetylated [61] The present results show that 14S RNA injected oocytes give rise to N-acetylated αA_2-crystallin chains The same is true of 14S RNA directed αA_2-crystallin synthesis in the reticulocyte lysate (section 4.4 4.) Three possible acetylating mechanisms in the oocytes may be considered

1 The 14S messenger, which has an estimated molecular weight of 360 000 (section 3.2.), is large enough to code for two polypeptide chains each of 20,000 molecular weight One of these two chains may be an acetylating enzyme or the αA_2 polypeptide may itself have acetylating properties

2. The 14S messenger may require initiation with acetyl-Met-tRNA$_f^{Met}$.
3. Acetylation takes place after initiation with Met-tRNA$_f^{Met}$ and is determined by the N-terminal amino acid sequence.

The first possibility is rendered unlikely by the observation that 10S RNA from lens directs the synthesis of N-acetylated α-crystallin B chains in a reticulocyte cell-free system (section 4.5.3.), whilst the amino acid sequences of the B chains are, with exception of the N-termini, different to those of the A chains.

The second possibility is unlikely as for the analogous situation in the reticulocyte system[69] and the lens system[151] no acetylated Met-tRNA$_f^{Met}$ was found.

The most obvious explanation is, therefore, again, that the protein synthesizing machinery of different tissues, from different species, possesses an acetylation mechanism which is able to recognize and acetylate a certain amino acid sequence.

The results reported here also show that calf lens αA_2-crystallin chains are stable, at all stages of assembly, in frog oocytes. Moreover the translation of the crystallin messenger and the stability of the products formed lend support to the idea that the oocyte system may prove to be a generally useful micro-assay for eukaryotic messengers.

Acrylamide	Union Chimique Belge
Amido Black	E Merck AG
ATP (dinatrium salt)	C F Boehringer & Soehne
unlabelled amino acids	Sigma
labelled amino acids	The Radiochemical Centre, Amersham
Coomassie blue	Edward Gurr Ltd
creatine phosphate	C F Boehringer & Soehne
creatine phosphokinase	C F Boehringer & Soehne
diethyl pyrocarbonate	Fluka AG
DOC	E Merck AG
Dowex	Fluka AG
E coli tRNA	General Biochemicals
EDTA	E Merck AG
Ethylenimine	Fluka AG
GSH	E Merck AG
GTP (trilithium salt)	Fluka AG
2-mercaptoethanol	Koch Light Laboratories Ltd
N,N'methylene-bisacrylamide	Schuchardt, Munchen
phenol	E Merck AG
phosphoenolpyruvate (trisodium salt)	Sigma
Pronase P	Serva
pyruvate kinase	C F Boehringer & Soehne
RNase	C F Boehringer & Soehne
SDS	Sigma
Subtilisin	Nutritional Biochemical Corp
Sucrose	BDH Chemicals Ltd
Trypsin	Worthington Biochemical Corp

SUMMARY

In this thesis the isolation and translation of messenger RNA from calf lens is described

This system has been chosen for the isolation of messenger RNA, as the lens is one of those unique tissues which synthesize a small number of polypeptides in relatively high content

Polyribosomes were isolated from lenses of young calves From these polyribosomes the RNA was extracted and separated using a zonal rotor, which enables the fractionation of relatively large quantities of RNA with excellent resolution Using this technique two RNA fractions were obtained with sedimentation values of 10S and 14S They appeared to contain mainly the messengers for α-crystallin polypeptide chains

The molecular weight and the base composition of these messenger fractions were determined These RNAs could also be isolated from ribonucleoprotein particles, obtained by dissociation of the polyribosomes with EDTA The conformation of the 10S messenger fraction was studied by electron microscopy Both the 10S and 14S RNAs were able to stimulate the amino acid incorporation in a preincubated cell-free system The definite proof for their messenger function was given by their translation in heterologous systems the cell-free systems derived from rabbit reticulocytes and Krebs II ascites cells and the *in vivo* system from the frog Xenopus laevis

Addition of the 14S messenger to these systems resulted in the synthesis of exclusively A_2 chains of α-crystallin The 10S messenger fraction directed, besides the synthesis of some unidentified polypeptides, the synthesis of B chains of α-crystallin

The products were identified by SDS gel electrophoresis, electrophoresis on acidic and basic urea gels, and by analysis of the N-terminal peptides Of the product of the 14S messenger, the αA_2 chain, the methionine containing tryptic peptides were compared with the methionine peptides of native αA_2 chains All these analyses confirmed the similarity between the newly synthesized chains and the native polypeptides

It further appeared from this investigation that

1 no lens specific factors are needed for the translation of the lens messengers in the heterologous systems described This observation supports the assertion that for the translation of messengers tissue specific initiation factors are not obligatory

2 the methionine residues, present at the N-termini of all α-crystallin polypeptide chains, are derived from the initiator Met-tRNA
3 both the reticulocyte system and the oocyte are able to acetylate the N-termini of the α-crystallin polypeptide chains This led us to speculate that every eukaryotic system possesses an acetylation mechanism ribosome bound or free in the cytoplasm, which is able to recognize and acetylate a certain amino acid sequence
4 there is no messenger present in our RNA preparations, which is able to prime the synthesis of α-crystallin A_1 chains This observation sustains the postulation of Palmer and Papaconstantinou, that the A_1 chain of α-crystallin is not synthesized as the result of direct genetic expression It is not excluded, however, that a messenger for αA_1 is present, but that its translation is blocked

In dit proefschrift wordt de isolatie en translatie van boodschapper RNA uit kalfslenzen beschreven.

De keuze van een dergelijk systeem voor de isolatie van boodschapper RNA, vindt zijn oorsprong in het feit dat de ooglens één van die unieke organen is die slechts een beperkt aantal eiwitten in hoog percentage synthetiseren.

Uit de lenzen van jonge kalveren werden de polyribosomen geisoleerd, die als uitgangsmateriaal dienden voor de bereiding van boodschapper RNA. Bij de isolatie van RNA uit polyribosomen werd gebruik gemaakt van een zonale rotor, die het mogelijk maakte met een hoog oplossend vermogen relatief grote hoeveelheden RNA te fractioneren.

Met behulp van deze techniek werden twee RNA-fracties verkregen, die voornamelijk de boodschappers voor α-crystalline-polypeptideketens bevatten. Eén van de RNA-fracties had een sedimentatiewaarde van ± 10S, terwijl de ander een sedimentatiewaarde van ± 14S vertoonde. Van deze boodschappers werd het molekulair gewicht en de base-samenstelling bepaald. Voorts werd aangetoond dat identieke RNA-fracties verkregen konden worden uit ribonucleoproteine-partikels, na dissociatie van de polyribosomen met behulp van EDTA. De 10S boodschapper-fractie werd ook met behulp van de elektronenmikroskoop onderzocht. Zowel de 10S als de 14S boodschapper-fractie bleek in staat de aminozuurincorporatie in een gepreincubeerd cel-vrij systeem te stimuleren.

Het definitieve bewijs voor de boodschapperfunktie van deze 10S en 14S RNA-fracties werd geleverd door hun translatie in heterologe systemen, zoals de cel-vrije systemen verkregen uit konijn-reticulocyten en uit Krebs II ascites-cellen en het *in vivo*-systeem van de oocyt van de klauwpad Xenopus laevis. Toevoegen van de 14S boodschapper-fractie aan deze drie systemen leidde tot de synthese van uitsluitend A_2-ketens van α-crystalline, terwijl de 10S boodschapper-fractie naast enkele, nog niet nader geidentificeerde polypeptiden, B-ketens van α-crystalline synthetiseerde.

De produkten werden geidentificeerd door middel van gel-electroforese in SDS-gels, die scheiden op basis van molekulair gewicht, en in zure en basische ureum-gels die scheiden op basis van lading. Voorts werden de N-terminale sequenties geanalyseerd, terwijl van het produkt van de 14S boodschapper bovendien de methionine-bevattende tryptische peptiden werden

vergeleken met de methionine-peptiden van natieve αA_2-ketens Al deze analyses toonden een volledige overeenkomst aan tussen de nieuw gesynthetiseerde ketens en de natieve ketens

Verder bleek uit dit onderzoek, dat

1 voor de translatie van deze lens-boodschapper in de genoemde heterologe systemen geen lens-specifieke factoren vereist waren, hetgeen de stelling ondersteunt, dat voor de translatie van messengers niet per se cel-specifieke of soort-specifieke factoren vereist zijn
2 het methionine-residue, aanwezig op de N-terminale plaats van alle α-crystalline-ketens, afkomstig is van het initiator methionyl-tRNA
3 zowel het reticulocyten-systeem als de oocyt in staat zijn α-crystalline (poly)peptide-ketens N-terminaal te acetyleren, zoals dat ook in de lens zelf gebeurt Deze waarneming vormde de achtergrond van de hypothese dat ieder eukaryotisch systeem een acetyleringsmechanisme bezit, dat in staat is een bepaalde aminozuurvolgorde te herkennen en te acetyleren
4 er geen boodschapper aanwezig was die aanleiding gaf tot de synthese van αA_1-ketens, hetgeen het postulaat ondersteunt dat de synthese van αA_1-ketens niet het directe gevolg is van genetische expressie Dat geen van de geïsoleerde boodschappers aanleiding gaf tot de synthese van αA_1-ketens sluit overigens het aanwezig zijn van dit boodschapper-RNA niet geheel uit Het zou denkbaar zijn dat translatie van deze boodschapper geblokkeerd is

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STELLINGEN

1.

Ten onrechte wordt door Blobel de methode van Gierer gebruikt voor de bepaling van het molekulairgewicht van reticulocyte messenger RNA.

G. Blobel, Proc.Natl.Acad.Sci. U.S., **68**, 832 (1971).

2.

De methode om messenger RNA te extraheren met behulp van opeenvolgende phenolextracties bij verschillende pH, zoals beschreven door Brawerman en medewerkers, is aan bedenkingen onderhevig.

G. Brawerman, J. Mendecki en S.Y. Lee, Biochem., **11**, 637 (1972).

3.

Bij het kinetisch onderzoek naar de regeneratie van rhodopsine zoals beschreven door Plante en Rabinovitch worden aspecifieke reacties van het 11-cis retinaldehyde met het onzuivere opsine-preparaat niet in beschouwing genomen.

E.O. Plante en B. Rabinovitch, Biochem.Biophys.Res.Commun., **46**, 725 (1972).

4.

Door Holland en medewerkers is de aanwezigheid van proteolytische enzymen in virussen onvoldoende aangetoond.

J.J. Holland, M. Doyle, J. Perrault, D T. Kingsbury en J. Etchison, Biochem.Biophys.Res.Commun., **46**, 634 (1972).

5.

Men dient er rekening mee te houden dat het zuiveren en reconstitueren van eiwit-synthetiserende cel-vrije systemen de specificiteit van messenger-herkenning kan beinvloeden.

6.

Dat het Endonuclease I een belangrijke rol speelt bij de werking van het Colicine E_2 is door Almendinger en Hager onvoldoende aangetoond.

R. Almendinger en L.P. Hager, Nature, New Biology, **235**, 199 (1972).

7.

De identiteit van het door Olofson en medewerkers geisoleerde tetraalkoxyetheenderivaat uit 4,5 tetramethyl 1,3 dioxoleniumfluoroboraat dient betwijfeld te worden.

R.A. Olofson, S.W. Walinsky, J.P. Marino en J.L. Jernow, J.Am.Chem.Soc., **90**, 6554 (1968).

8.

Door de in vele natuurgebieden plaatsvindende motorcrosses te concentreren in enkele, weinig kwetsbare gebieden zou een waardevolle bijdrage geleverd worden tot het natuurbehoud.

9.

De manier waarop Rijksinstellingen subsidie verlenen voor het drukken van dissertaties doet de indruk ontstaan dat de hoogte van de drukkosten een maatstaf is voor de wetenschappelijke relevantie van een proefschrift.

A.J.M. Berns
22 juni 1972